EXHIBIT AA

US009079934B2

(12) **United States Patent**
Watanabe et al.

(10) **Patent No.: US 9,079,934 B2**
(45) **Date of Patent: Jul. 14, 2015**

(54) **ANTISENSE NUCLEIC ACIDS**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **13/819,520**

(22) PCT Filed: **Aug. 31, 2011**

(86) PCT No.: **PCT/JP2011/070318**
§ 371 (c)(1),
(2), (4) Date: **Apr. 10, 2013**

(87) PCT Pub. No.: **WO2012/029986**
PCT Pub. Date: **Mar. 8, 2012**

(65) **Prior Publication Data**

US 2013/0211062 A1 Aug. 15, 2013

(30) **Foreign Application Priority Data**

Sep. 1, 2010 (JP) 2010-196032

(51) **Int. Cl.**

C07H 21/02	(2006.01)
C07H 21/04	(2006.01)
A61K 31/70	(2006.01)
C12N 15/11	(2006.01)
C12N 15/113	(2010.01)
C07H 21/00	(2006.01)
C12Q 1/68	(2006.01)

(52) **U.S. Cl.**
CPC ***C07H 21/04*** (2013.01); ***C07H 21/00*** (2013.01); ***C12N 15/111*** (2013.01); ***C12N 15/113*** (2013.01); *C12N 2310/11* (2013.01); *C12N 2310/315* (2013.01); *C12N 2310/321* (2013.01); *C12N 2310/3525* (2013.01); *C12N 2320/33* (2013.01)

(58) **Field of Classification Search**
None
See application file for complete search history.

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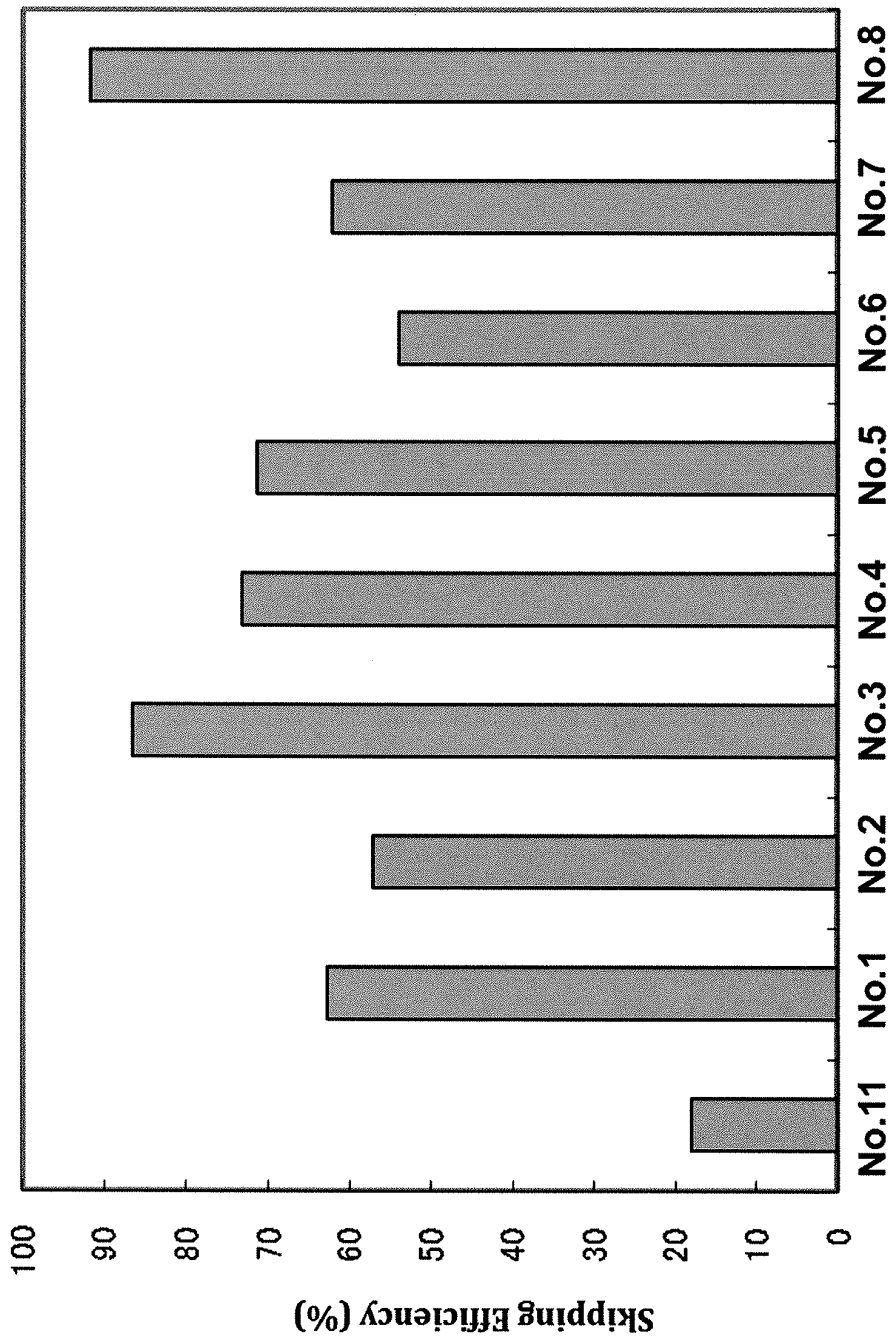
Primary Examiner — Sean McGarry
(74) *Attorney, Agent, or Firm* — Drinker Biddle & Reath LLP

(57) **ABSTRACT**

The present invention provides an oligomer which efficiently enables to cause skipping of the 53rd exon in the human dystrophin gene. Also provided is a pharmaceutical composition which causes skipping of the 53rd exon in the human dystrophin gene with a high efficiency.

7 Claims, 19 Drawing Sheets

Figure 1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No.7
No.6
No.5
No.4
No.3
No.2
No.1
No.11
100
90
80
70
60
50
40
30
20
10
0
Skipping Efficiency (%)

Figure 2

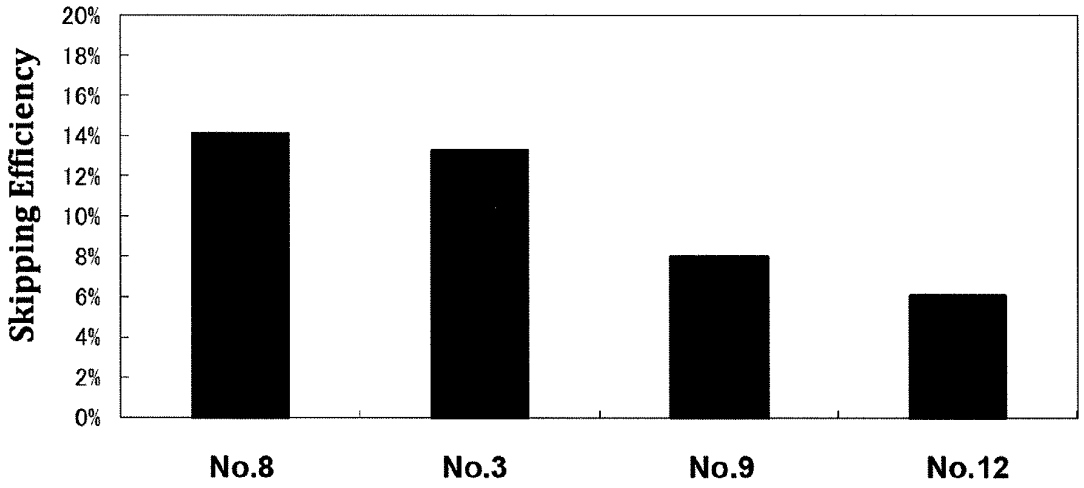
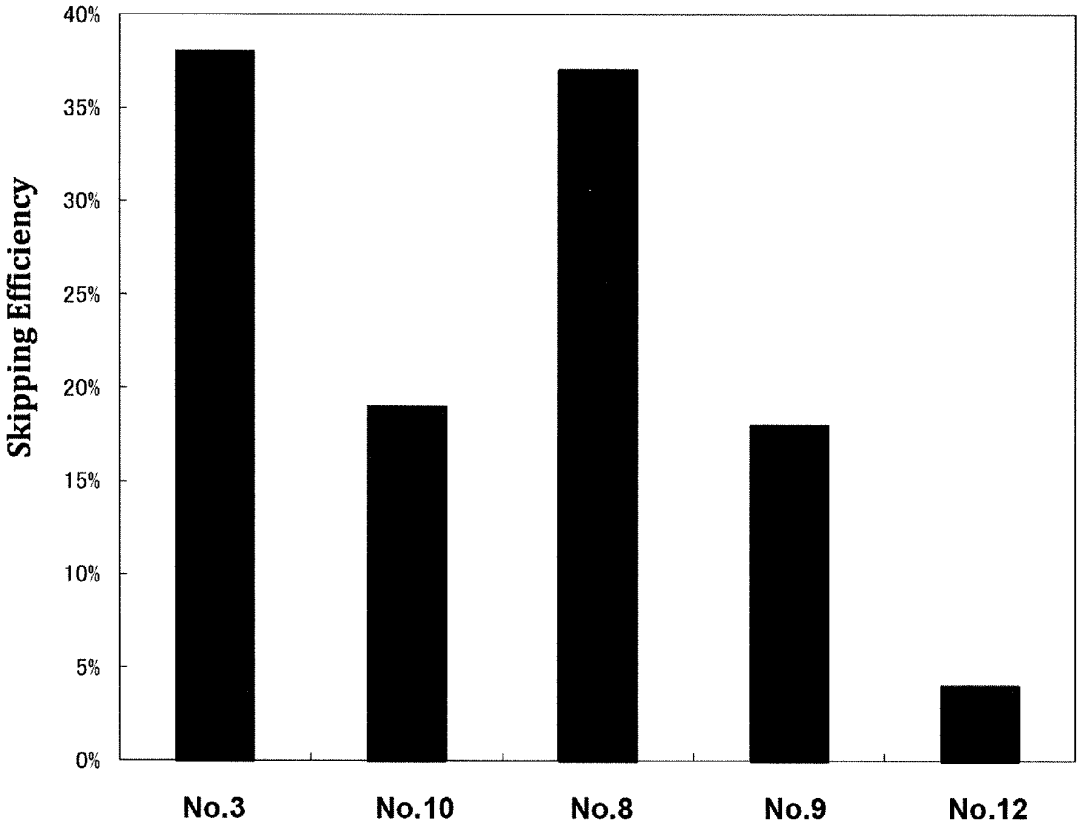


Figure 3


Skipping Efficiency
40%
35%
30%
25%
20%
15%
10%
5%
0%
No.3
No.10
No.8
No.9
No.12

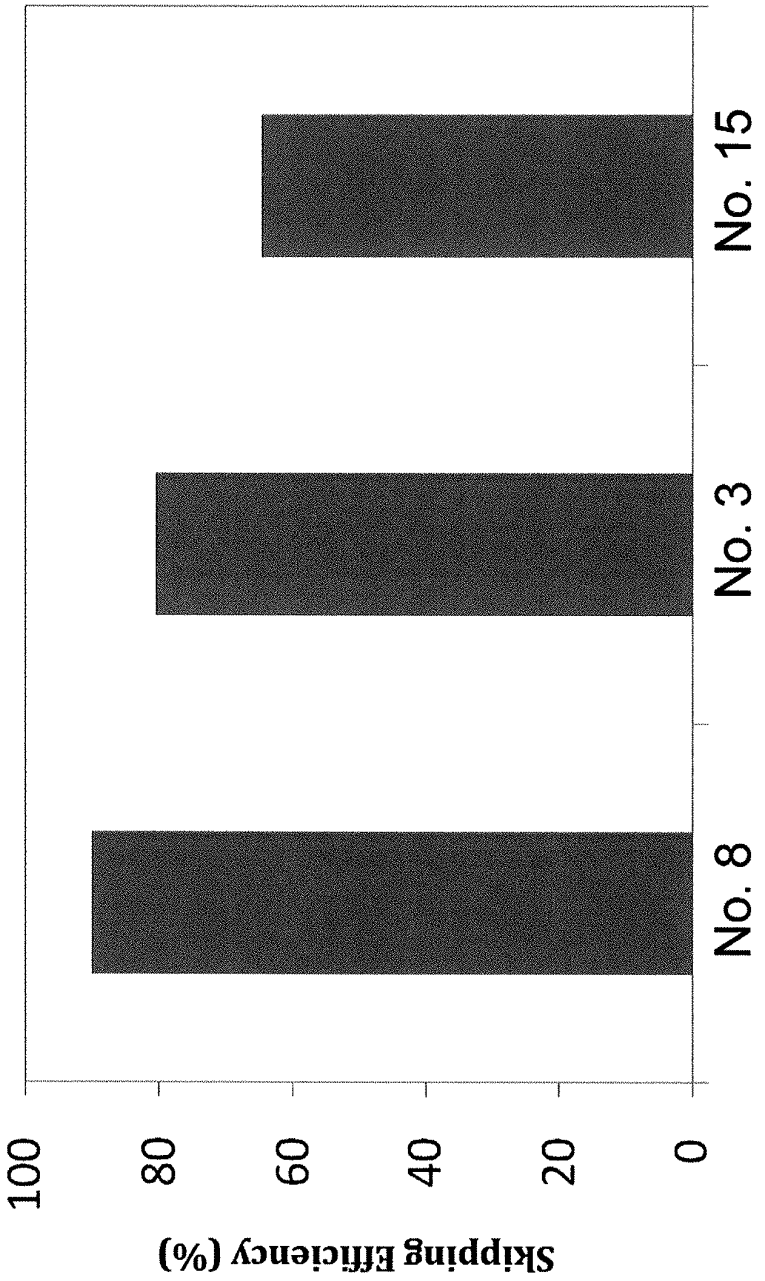


Figure 4

Figure 5

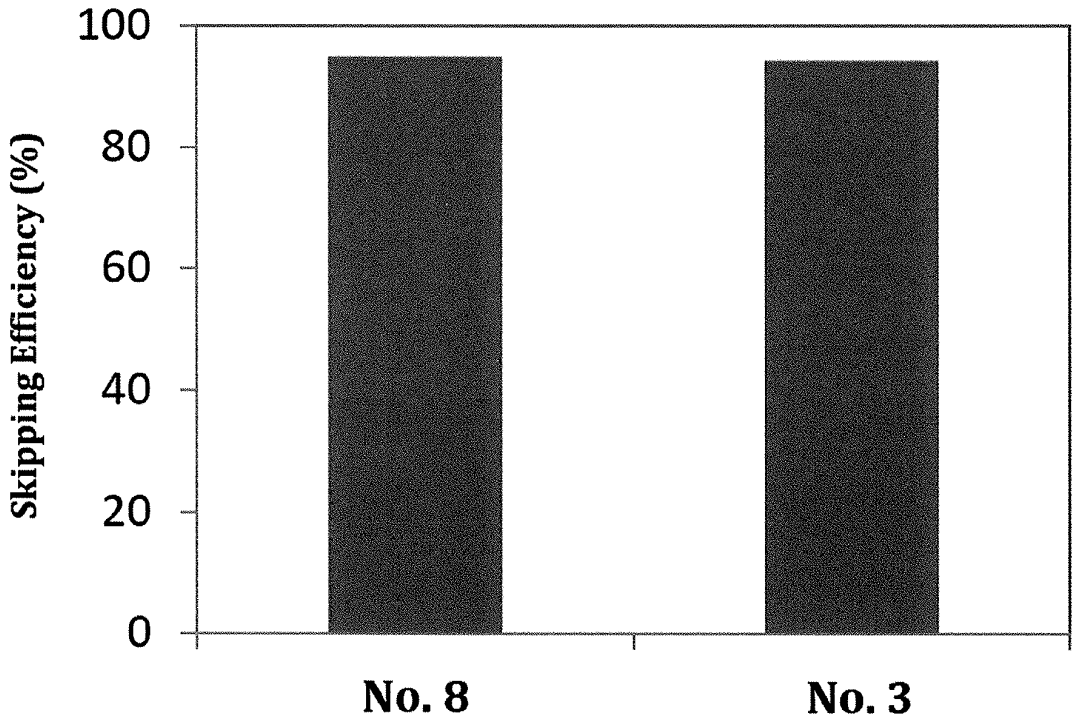


Figure 6

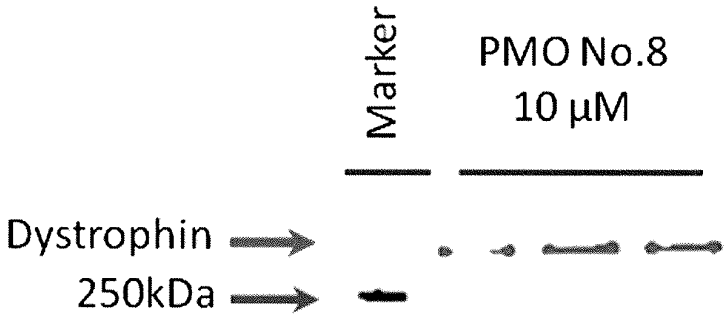


Figure 7

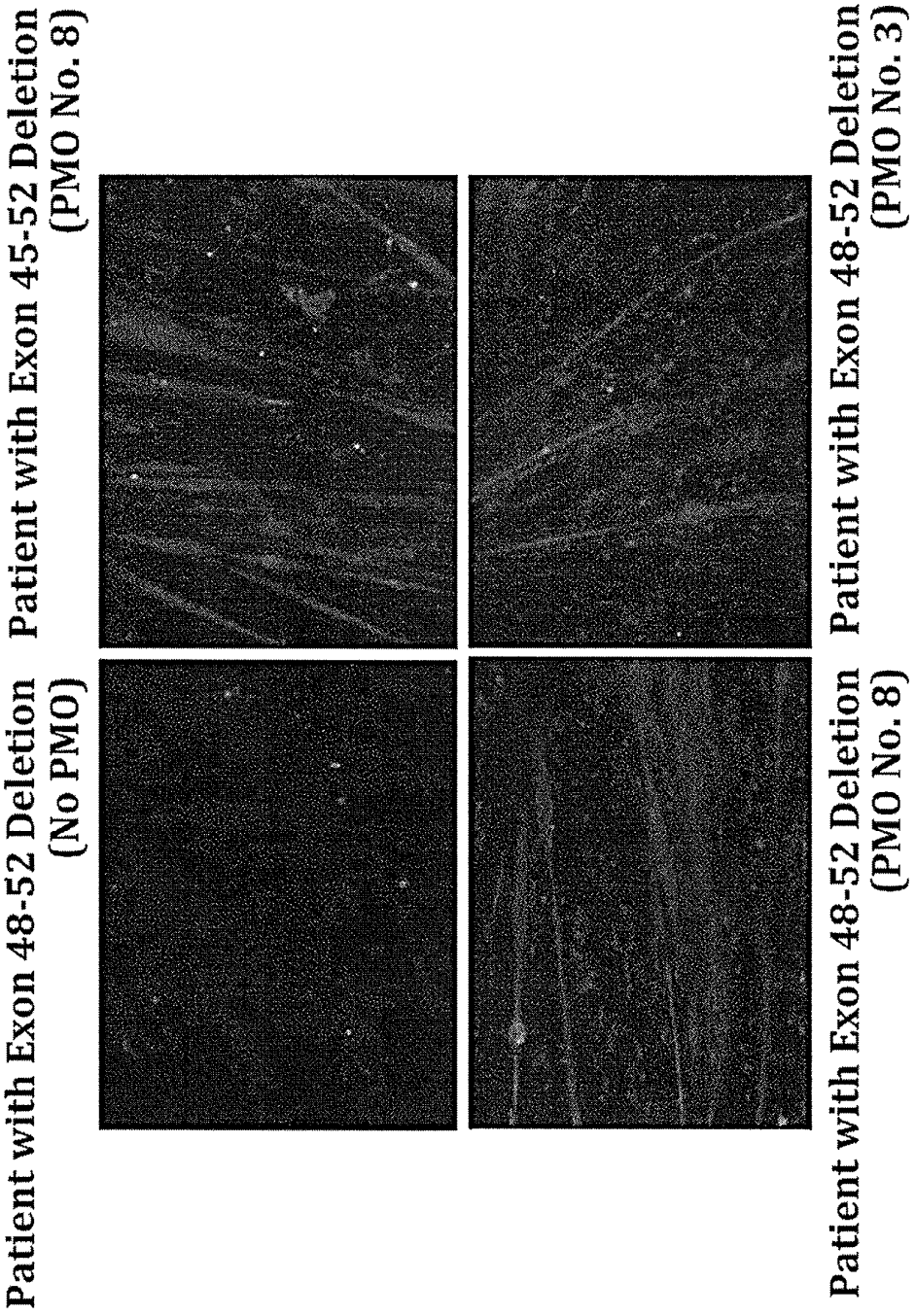
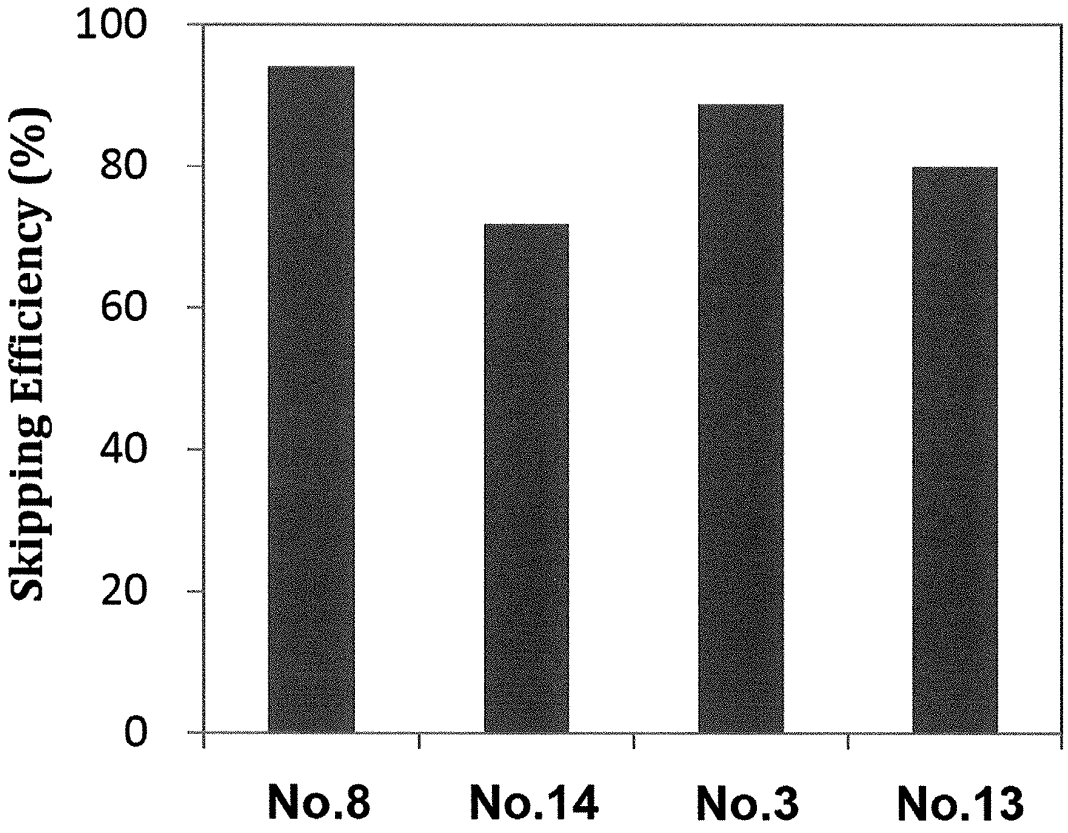

Patient with Exon 45-52 Deletion (PMO No. 8)
Patient with Exon 48-52 Deletion (PMO No. 3)
Patient with Exon 48-52 Deletion (No PMO)
Patient with Exon 48-52 Deletion (PMO No. 8)

Figure 8



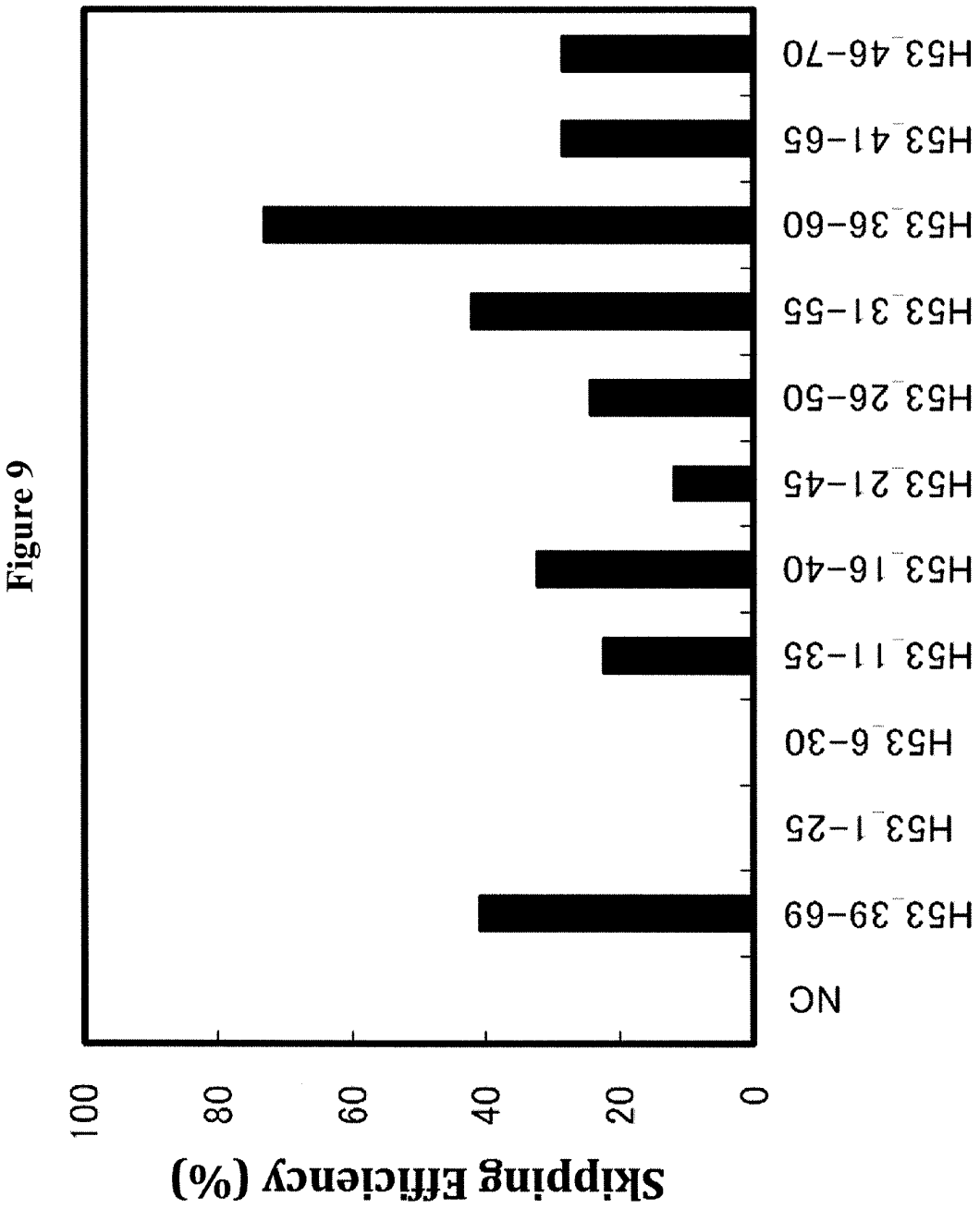

Skipping Efficiency (%)
100
80
60
40
20
0
NC
H53_39-69
H53_1-25
H53_6-30
H53_11-35
H53_16-40
H53_21-45
H53_26-50
H53_31-55
H53_36-60
H53_41-65
H53_46-70

Figure 9

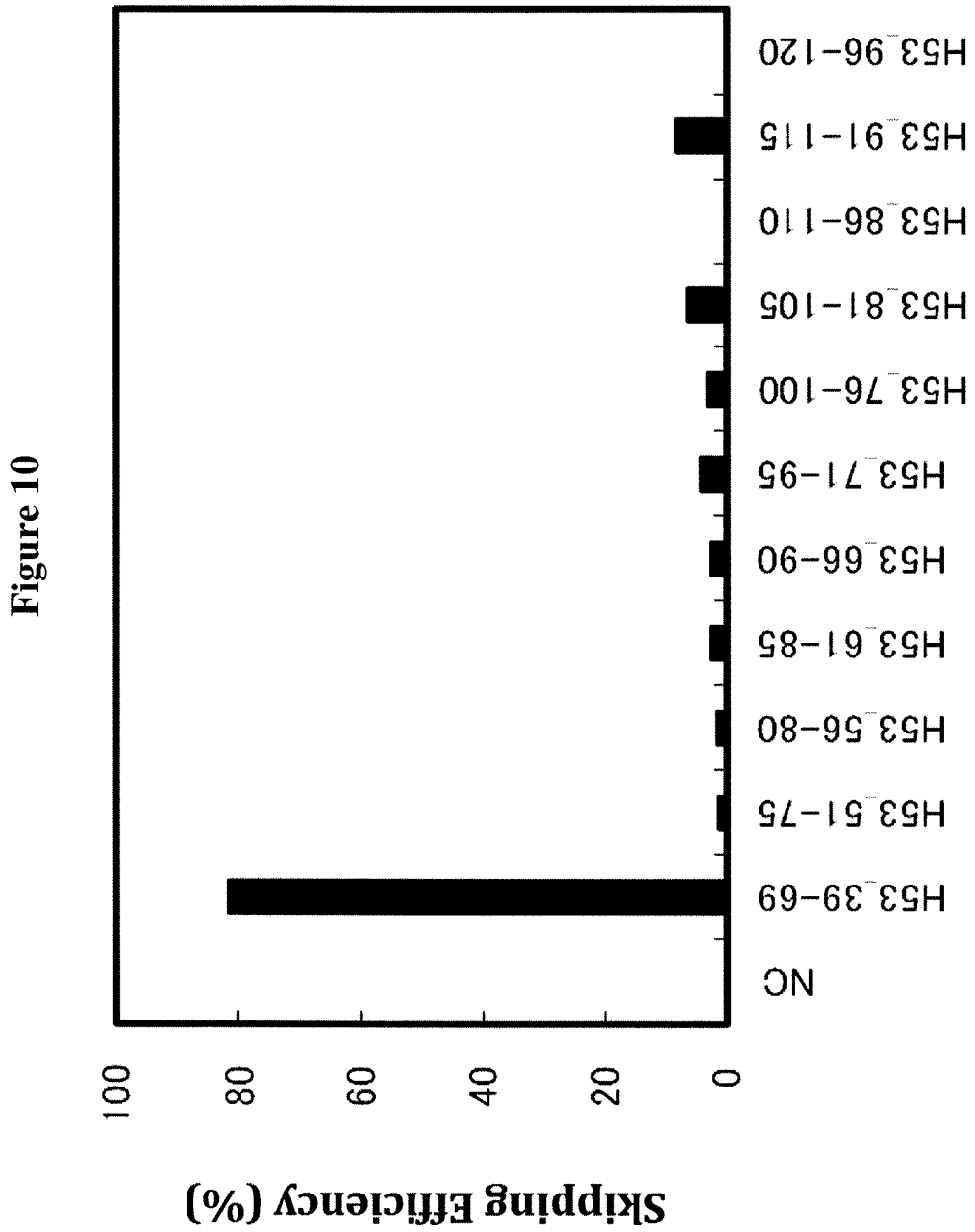

Skipping Efficiency (%)
100
80
60
40
20
0
NC
H53_39-69
H53_51-75
H53_56-80
H53_61-85
H53_66-90
H53_71-95
H53_76-100
H53_81-105
H53_86-110
H53_91-115
H53_96-120

Figure 10

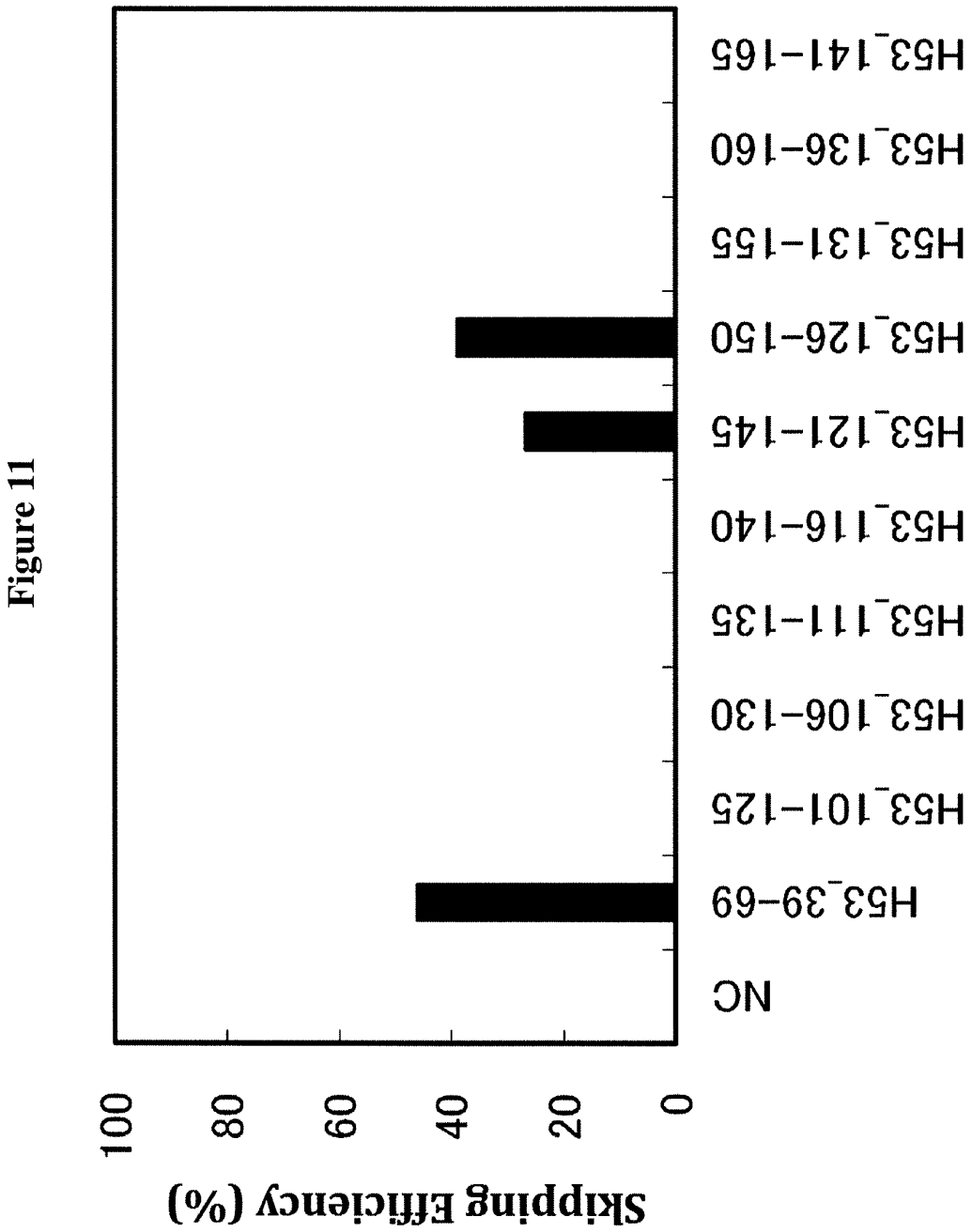

Skipping Efficiency (%)
0
20
40
60
80
100
NC
H53_39-69
H53_101-125
H53_106-130
H53_111-135
H53_116-140
H53_121-145
H53_126-150
H53_131-155
H53_136-160
H53_141-165

Figure 11

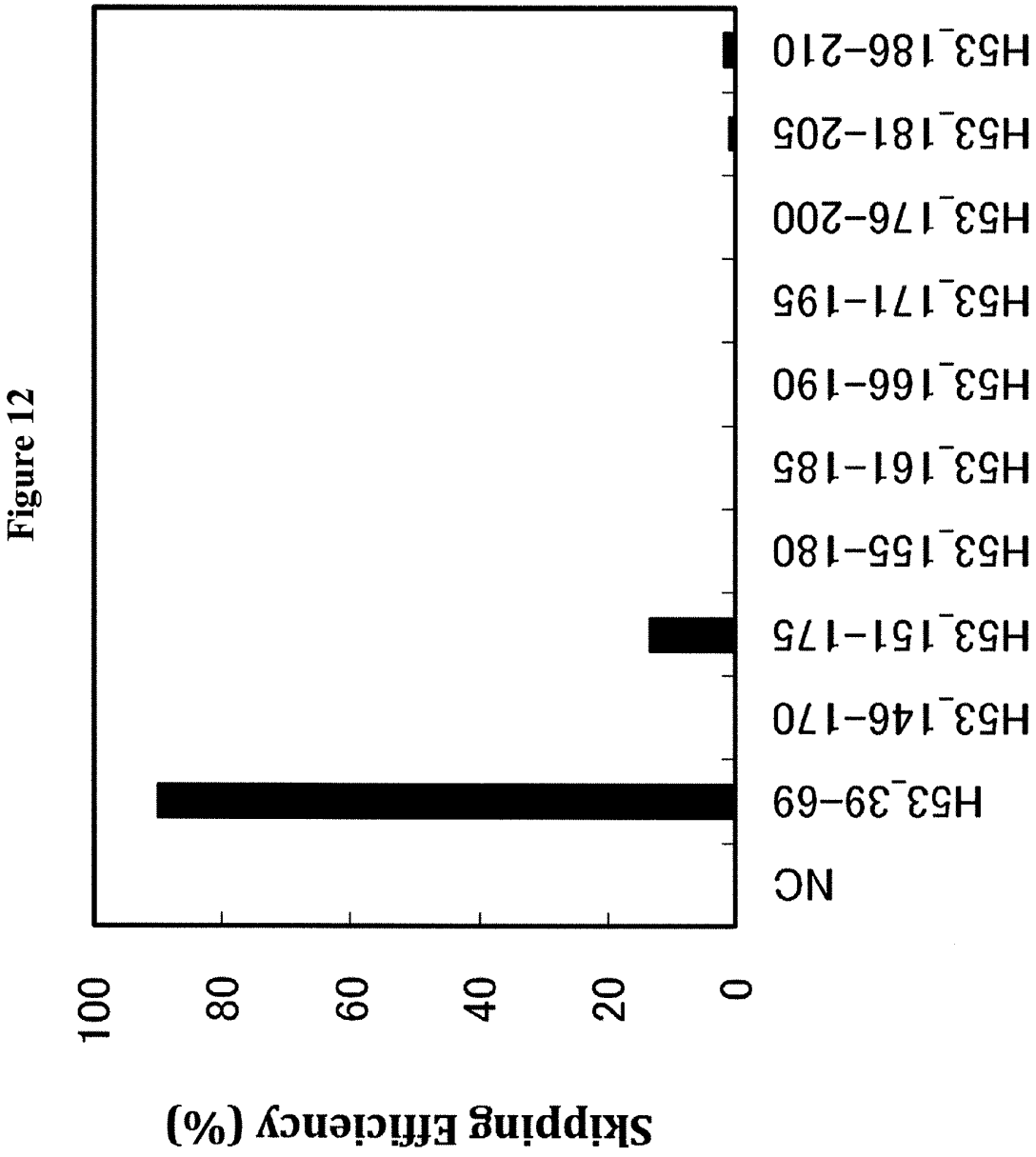

NC
H53_39-69
H53_146-170
H53_151-175
H53_155-180
H53_161-185
H53_166-190
H53_171-195
H53_176-200
H53_181-205
H53_186-210
0
20
40
60
80
100
Skipping Efficiency (%)

Figure 12

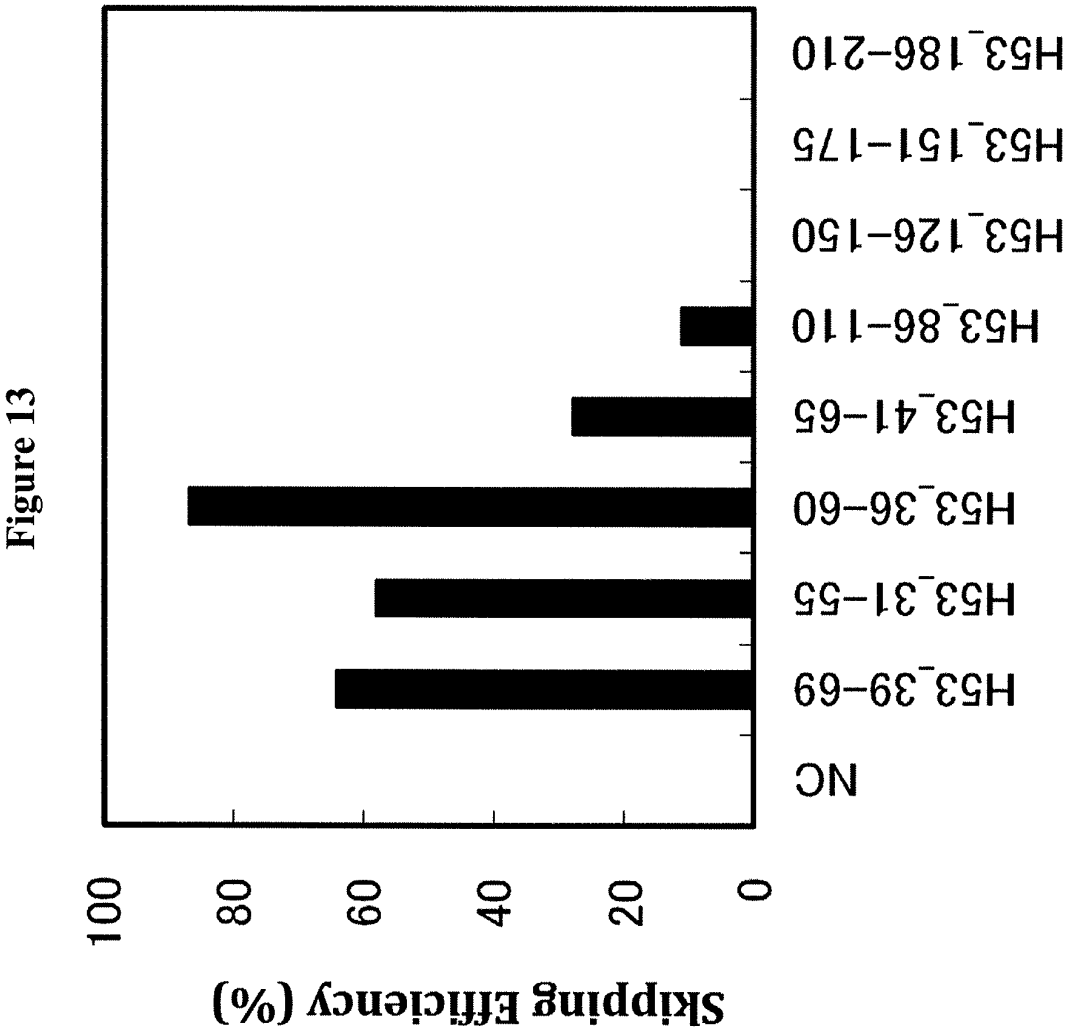
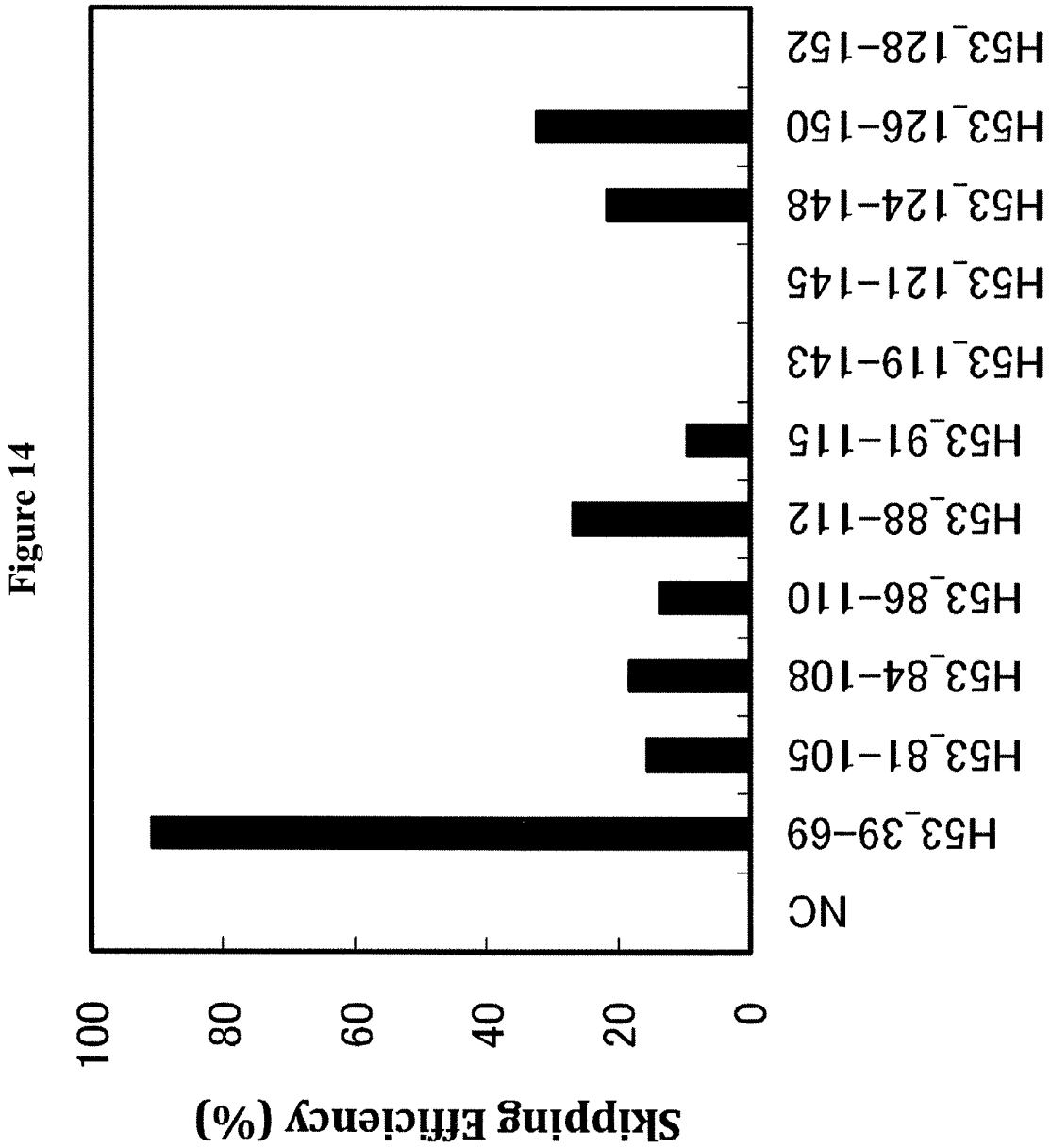

Skipping Efficiency (%)
0
20
40
60
80
100
NC
H53_39-69
H53_31-55
H53_36-60
H53_41-65
H53_86-110
H53_126-150
H53_151-175
H53_186-210

Figure 13


Skipping Efficiency (%)
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20
40
60
80
100
NC
H53_39-69
H53_81-105
H53_84-108
H53_86-110
H53_88-112
H53_91-115
H53_119-143
H53_121-145
H53_124-148
H53_126-150
H53_128-152

Figure 14

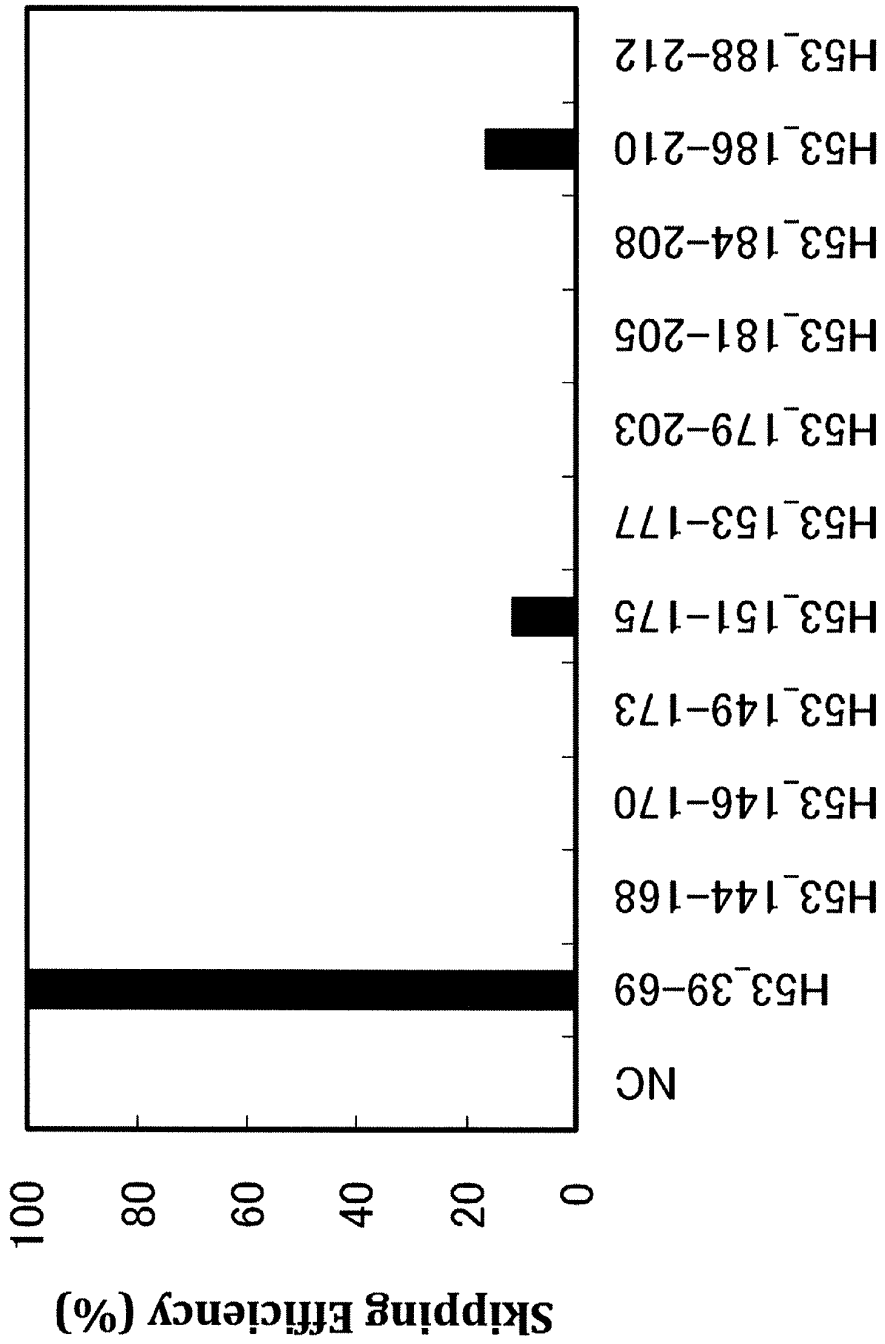

Skipping Efficiency (%)
100
80
60
40
20
0
NC
H53_39-69
H53_144-168
H53_146-170
H53_149-173
H53_151-175
H53_153-177
H53_179-203
H53_181-205
H53_184-208
H53_186-210
H53_188-212

Figure 15

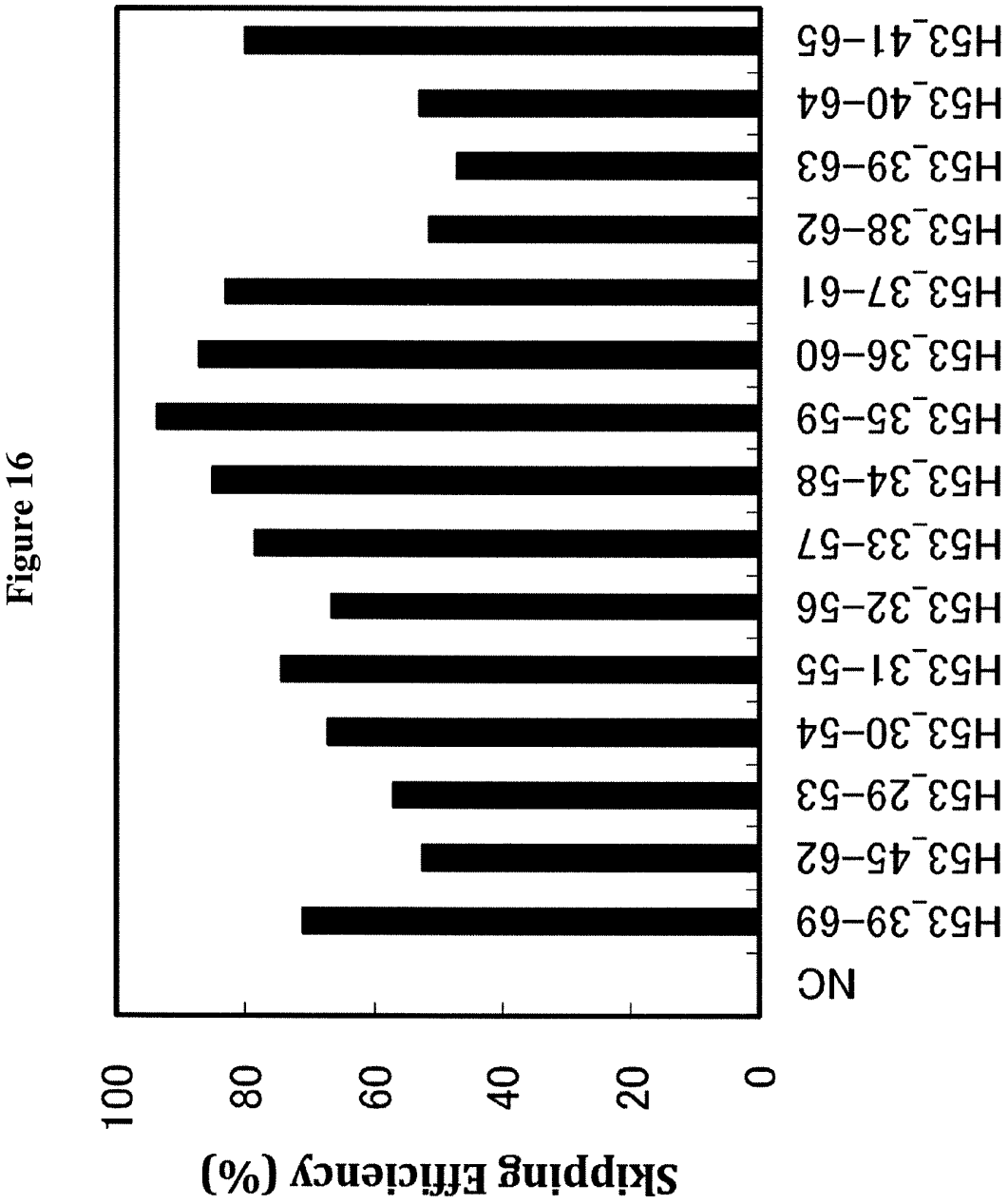
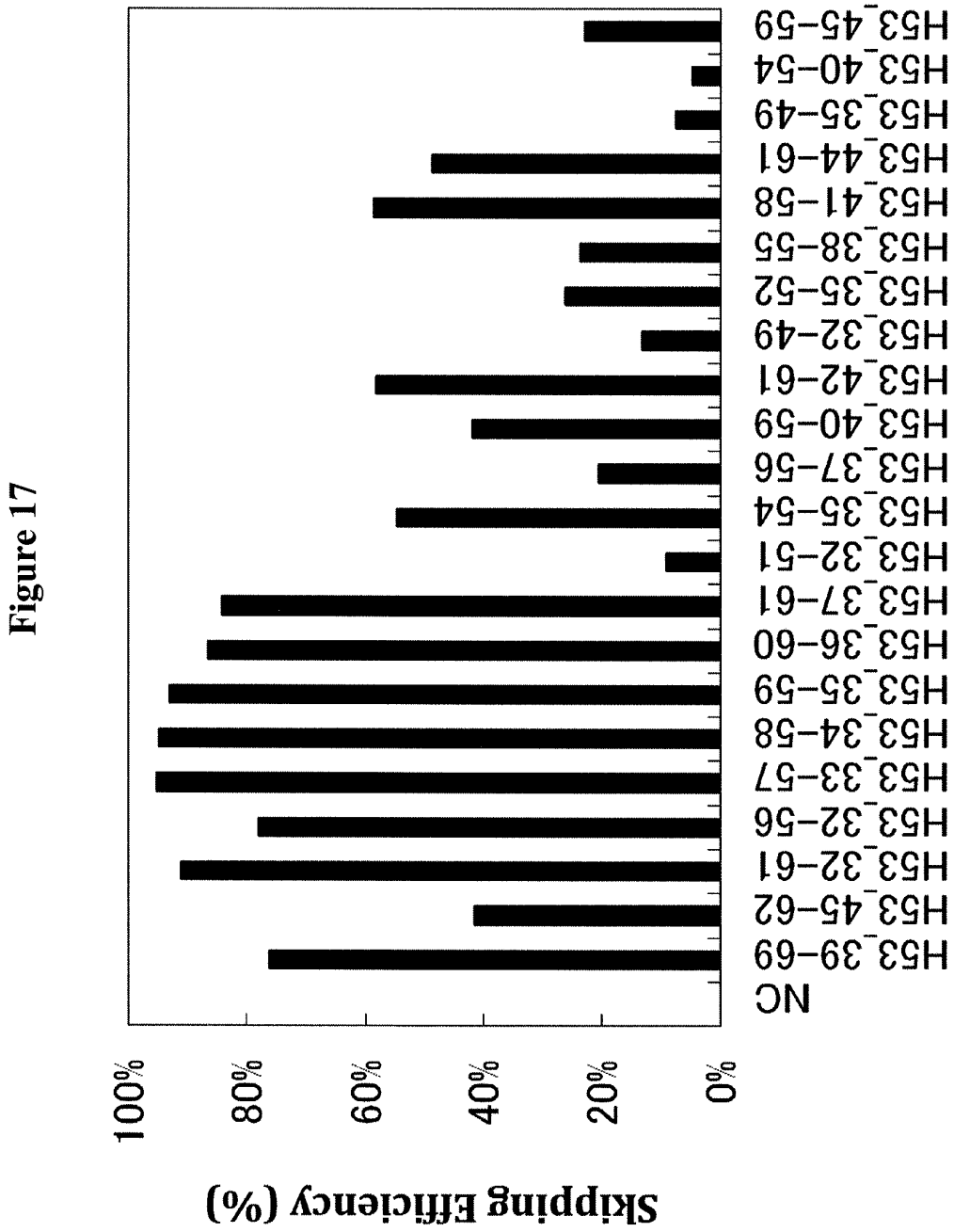

Skipping Efficiency (%)
0
20
40
60
80
100
NC
H53_39-69
H53_45-62
H53_29-53
H53_30-54
H53_31-55
H53_32-56
H53_33-57
H53_34-58
H53_35-59
H53_36-60
H53_37-61
H53_38-62
H53_39-63
H53_40-64
H53_41-65

Figure 16


Skipping Efficiency (%)
100%
80%
60%
40%
20%
0%
NC
H53_39-69
H53_45-62
H53_32-61
H53_32-56
H53_33-57
H53_34-58
H53_35-59
H53_36-60
H53_37-61
H53_32-51
H53_35-54
H53_37-56
H53_40-59
H53_42-61
H53_32-49
H53_35-52
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H53_41-58
H53_44-61
H53_35-49
H53_40-54
H53_45-59

Figure 17

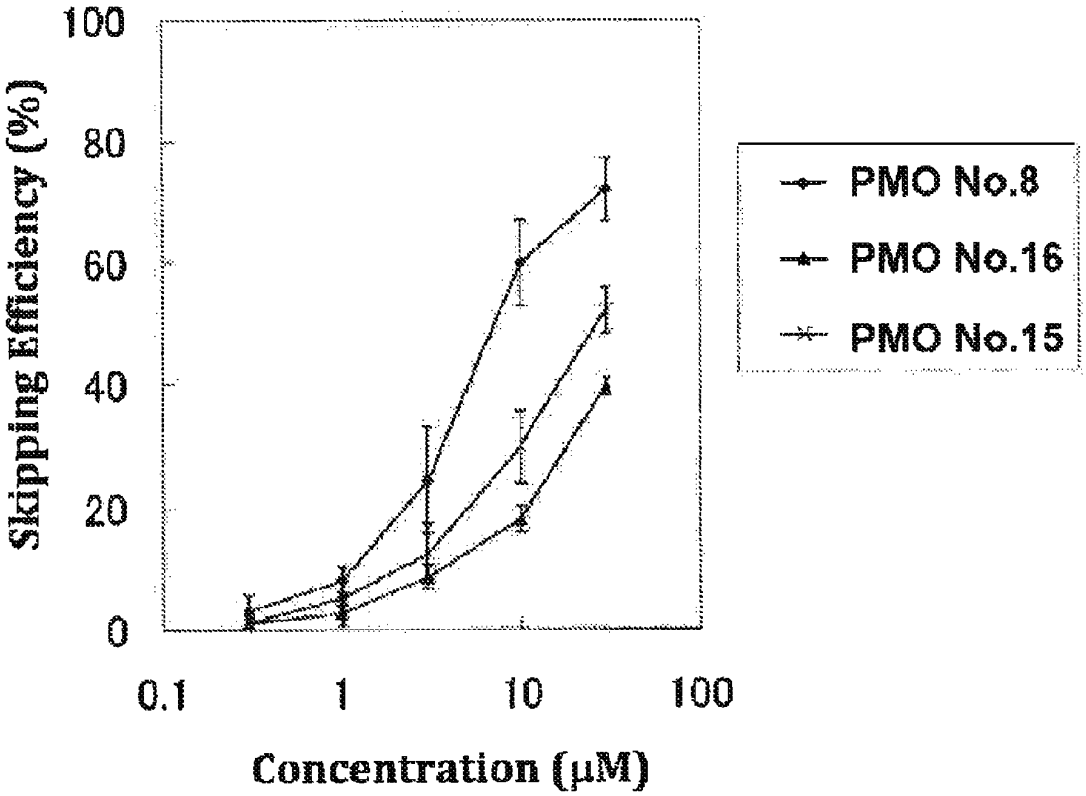

100
80
60
40
20
0
Skipping Efficiency (%)
0.1
1
10
100
Concentration (μM)
PMO No.8
PMO No.16
PMO No.15

Figure 18

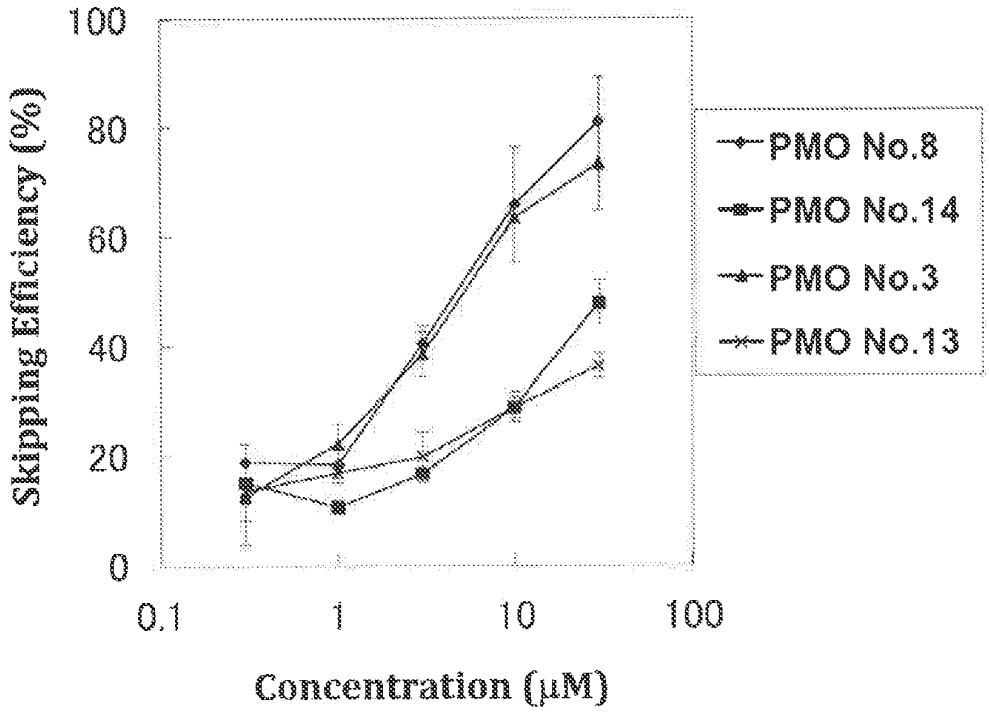

100
80
60
40
20
0
Skipping Efficiency (%)
0.1
1
10
100
Concentration (μM)
PMO No.8
PMO No.14
PMO No.3
PMO No.13

Figure 19

ANTISENSE NUCLEIC ACIDS

CROSS REFERENCE TO RELATED APPLICATIONS

This application is the National Stage of International Application No. PCT/JP2011/070318, filed Aug. 31, 2011, and claims benefit of Japanese Application No. 2010-196032, filed on Sep. 1, 2010, all of which are herein incorporated by reference in their entirety.

SEQUENCE LISTING

The instant application contains a sequence listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Mar. 29, 2013, is named G12__0074_Seq_Listing_revised_Sq_No__64.txt and is 24,294 bytes in size.

TECHNICAL FIELD

The present invention relates to an antisense oligomer which causes skipping of exon 53 in the human dystrophin gene, and a pharmaceutical composition comprising the oligomer.

BACKGROUND ART

Duchenne muscular dystrophy (DMD) is the most frequent form of hereditary progressive muscular dystrophy that affects one in about 3,500 newborn boys. Although the motor functions are rarely different from healthy humans in infancy and childhood, muscle weakness is observed in children from around 4 to 5 years old. Then, muscle weakness progresses to the loss of ambulation by about 12 years old and death due to cardiac or respiratory insufficiency in the twenties. DMD is such a severe disorder. At present, there is no effective therapy for DMD available, and it has been strongly desired to develop a novel therapeutic agent.

DMD is known to be caused by a mutation in the dystrophin gene. The dystrophin gene is located on X chromosome and is a huge gene consisting of 2.2 million DNA nucleotide pairs. DNA is transcribed into mRNA precursors, and introns are removed by splicing to synthesize mRNA in which 79 exons are joined together. This mRNA is translated into 3,685 amino acids to produce the dystrophin protein. The dystrophin protein is associated with the maintenance of membrane stability in muscle cells and necessary to make muscle cells less fragile. The dystrophin gene from patients with DMD contains a mutation and hence, the dystrophin protein, which is functional in muscle cells, is rarely expressed. Therefore, the structure of muscle cells cannot be maintained in the body of the patients with DMD, leading to a large influx of calcium ions into muscle cells. Consequently, an inflammation-like response occurs to promote fibrosis so that muscle cells can be regenerated only with difficulty.

Becker muscular dystrophy (BMD) is also caused by a mutation in the dystrophin gene. The symptoms involve muscle weakness accompanied by atrophy of muscle but are typically mild and slow in the progress of muscle weakness, when compared to DMD. In many cases, its onset is in adulthood. Differences in clinical symptoms between DMD and BMD are considered to reside in whether the reading frame for amino acids on the translation of dystrophin mRNA into the dystrophin protein is disrupted by the mutation or not (Non-Patent Document 1). More specifically, in DMD, the presence of mutation shifts the amino acid reading frame so that the expression of functional dystrophin protein is abolished, whereas in BMD the dystrophin protein that functions, though imperfectly, is produced because the amino acid reading frame is preserved, while a part of the exons are deleted by the mutation.

Exon skipping is expected to serve as a method for treating DMD. This method involves modifying splicing to restore the amino acid reading frame of dystrophin mRNA and induce expression of the dystrophin protein having the function partially restored (Non-Patent Document 2). The amino acid sequence part, which is a target for exon skipping, will be lost. For this reason, the dystrophin protein expressed by this treatment becomes shorter than normal one but since the amino acid reading frame is maintained, the function to stabilize muscle cells is partially retained. Consequently, it is expected that exon skipping will lead DMD to the similar symptoms to that of BMD which is milder. The exon skipping approach has passed the animal tests using mice or dogs and now is currently assessed in clinical trials on human DMD patients.

The skipping of an exon can be induced by binding of antisense nucleic acids targeting either 5' or 3' splice site or both sites, or exon-internal sites. An exon will only be included in the mRNA when both splice sites thereof are recognized by the spliceosome complex. Thus, exon skipping can be induced by targeting the splice sites with antisense nucleic acids. Furthermore, the binding of an SR protein to an exonic splicing enhancer (ESE) is considered necessary for an exon to be recognized by the splicing mechanism. Accordingly, exon skipping can also be induced by targeting ESE.

Since a mutation of the dystrophin gene may vary depending on DMD patients, antisense nucleic acids need to be designed based on the site or type of respective genetic mutation. In the past, antisense nucleic acids that induce exon skipping for all 79 exons were produced by Steve Wilton, et al., University of Western Australia (Non-Patent Document 3), and the antisense nucleic acids which induce exon skipping for 39 exons were produced by Annemieke Aartsma-Rus, et al., Netherlands (Non-Patent Document 4).

It is considered that approximately 8% of all DMD patients may be treated by skipping the 53rd exon (hereinafter referred to as "exon 53"). In recent years, a plurality of research organizations reported on the studies where exon 53 in the dystrophin gene was targeted for exon skipping (Patent Documents 1 to 4; Non-Patent Document 5). However, a technique for skipping exon 53 with a high efficiency has not yet been established.

Patent Document 1: International Publication WO 2006/000057

Patent Document 2: International Publication WO 2004/048570

Patent Document 3: US 2010/0168212

Patent Document 4: International Publication WO 2010/048586

Non-Patent Document 1: Monaco A. P. et al., Genomics 1988; 2: p. 90-95

Non-Patent Document 2: Matsuo M., Brain Dev 1996; 18: p. 167-172

Non-Patent Document 3: Wilton S. D., et al., Molecular Therapy 2007: 15: p. 1288-96

Non-Patent Document 4: Annemieke Aartsma-Rus et al., (2002) Neuromuscular Disorders 12: S71-S77

Non-Patent Document 5: Linda J. Popplewell et al., (2010) Neuromuscular Disorders, vol. 20, no. 2, p. 102-10

DISCLOSURE OF THE INVENTION

Under the foregoing circumstances, antisense oligomers that strongly induce exon 53 skipping in the dystrophin gene and muscular dystrophy therapeutics comprising oligomers thereof have been desired.

As a result of detailed studies of the structure of the dystrophin gene, the present inventors have found that exon 53 skipping can be induced with a high efficiency by targeting the sequence consisting of the 32nd to the 56th nucleotides from the 5' end of exon 53 in the mRNA precursor (hereinafter referred to as "pre-mRNA") in the dystrophin gene with antisense oligomers. Based on this finding, the present inventors have accomplished the present invention.

That is, the present invention is as follows.

[1] An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences consisting of the 31st to the 53rd, the 31st to the 54th, the 31st to the 55th, the 31st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th, or the 36th to the 58th nucleotides, from the 5' end of the 53rd exon in the human dystrophin gene.

[2] The antisense oligomer according to [1] above, which is an oligonucleotide.

[3] The antisense oligomer according to [2] above, wherein the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified.

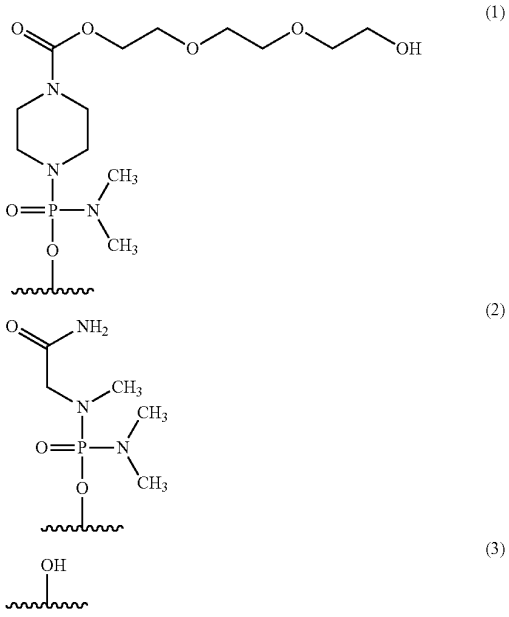
[4] The antisense oligomer according to [3] above, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br and I (wherein R is an alkyl or an aryl and R' is an alkylene).

[5] The antisense oligomer according to [3] or [4] above, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond and a boranophosphate bond.

[6] The antisense oligomer according to [1] above, which is a morpholino oligomer.

[7] The antisense oligomer according to [6] above, which is a phosphorodiamidate morpholino oligomer.

[8] The antisense oligomer according to any one of [1] to [7] above, wherein the 5' end is any one of the groups of chemical formulae (1) to (3) below:



[9] The antisense oligomer according to any one of [1] to [8] above, consisting of a nucleotide sequence complementary to the sequences consisting of the 32nd to the 56th or the 36th to the 56th nucleotides from the 5' end of the 53rd exon in the human dystrophin gene.

[10] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by any one selected from the group consisting of SEQ ID NOS: 2 to 37.

[11] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by any one selected from the group consisting of SEQ ID NOS: 11, 17, 23, 29 and 35.

[12] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by SEQ ID NO: 11 or 35.

[13] A pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the antisense oligomer according to any one of [1] to [12] above, or a pharmaceutically acceptable salt or hydrate thereof.

The antisense oligomer of the present invention can induce exon 53 skipping in the human dystrophin gene with a high efficiency. In addition, the symptoms of Duchenne muscular dystrophy can be effectively alleviated by administering the pharmaceutical composition of the present invention.

BRIEF DESCRIPTION OF DRAWINGS

FIG. **1** shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cell line (RD cells).

FIG. **2** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into human normal tissue-derived fibroblasts (TIG-119 cells) to induce differentiation into muscle cells.

FIG. **3** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into human DMD patient-derived fibroblasts (5017 cells) to induce differentiation into muscle cells.

FIG. **4** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52) to induce differentiation into muscle cells.

FIG. **5** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. **6** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. **7** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52 or deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. **8** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52) to induce differentiation into muscle cells.

FIG. **9** shows the efficiency of exon 53 skipping (2'-OMe-S—RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **10** shows the efficiency of exon 53 skipping (T-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **11** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **12** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **13** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **14** shows the efficiency of exon 53 skipping (T-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **15** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **16** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **17** shows the efficiency of exon 53 skipping (T-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **18** shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at the respective concentrations of the oligomers.

FIG. **19** shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at the respective concentrations of the oligomers.

BEST MODE FOR CARRYING OUT THE INVENTION

Hereinafter, the present invention is described in detail. The embodiments described below are intended to be presented by way of example merely to describe the invention but not limited only to the following embodiments. The present invention may be implemented in various ways without departing from the gist of the invention.

All of the publications, published patent applications, patents and other patent documents cited in the specification are herein incorporated by reference in their entirety. The specification hereby incorporates by reference the contents of the specification and drawings in the Japanese Patent Application (No. 2010-196032) filed Sep. 1, 2010, from which the priority was claimed.

1. Antisense Oligomer

The present invention provides the antisense oligomer (hereinafter referred to as the "oligomer of the present invention") which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences (hereinafter also referred to as "target sequences") consisting of the 31st to the 53rd, the 31st to the 54th, the 31st to the 55th, the 31st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th, or the 36th to the 58th nucleotides, from the 5' end of the 53rd exon in the human dystrophin gene.

[Exon 53 in Human Dystrophin Gene]

In the present invention, the term "gene" is intended to mean a genomic gene and also include cDNA, mRNA precursor and mRNA. Preferably, the gene is mRNA precursor, i.e., pre-mRNA.

In the human genome, the human dystrophin gene locates at locus Xp21.2. The human dystrophin gene has a size of 3.0 Mbp and is the largest gene among known human genes. However, the coding regions of the human dystrophin gene are only 14 kb, distributed as 79 exons throughout the human dystrophin gene (Roberts, R G., et al., Genomics, 16: 536-538 (1993)). The pre-mRNA, which is the transcript of the human dystrophin gene, undergoes splicing to generate mature mRNA of 14 kb. The nucleotide sequence of human wild-type dystrophin gene is known (GenBank Accession No. NM_004006).

The nucleotide sequence of exon 53 in the human wild-type dystrophin gene is represented by SEQ ID NO: 1.

The oligomer of the present invention is designed to cause skipping of exon 53 in the human dystrophin gene, thereby modifying the protein encoded by DMD type of dystrophin gene into the BMD type of dystrophin protein. Accordingly, exon 53 in the dystrophin gene that is the target of exon skipping by the oligomer of the present invention includes both wild and mutant types.

Specifically, exon 53 mutants of the human dystrophin gene include the polynucleotides defined in (a) or (b) below.

(a) A polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1; and,

(b) A polynucleotide consisting of a nucleotide sequence having at least 90% identity with the nucleotide sequence of SEQ ID NO: 1.

As used herein, the term "polynucleotide" is intended to mean DNA or RNA.

As used herein, the term "polynucleotide that hybridizes under stringent conditions" refers to, for example, a poly-

nucleotide obtained by colony hybridization, plaque hybridization, Southern hybridization or the like, using as a probe all or part of a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of, e.g., SEQ ID NO: 1. The hybridization method which may be used includes methods described in, for example, "Sambrook & Russell, Molecular Cloning: A Laboratory Manual Vol. 3, Cold Spring Harbor, Laboratory Press 2001," "Ausubel, Current Protocols in Molecular Biology, John Wiley & Sons 1987-1997," etc.

As used herein, the term "complementary nucleotide sequence" is not limited only to nucleotide sequences that form Watson-Crick pairs with target nucleotide sequences, but is intended to also include nucleotide sequences which form Wobble base pairs. As used herein, the term Watson-Crick pair refers to a pair of nucleobases in which hydrogen bonds are formed between adenine-thymine, adenine-uracil or guanine-cytosine, and the term Wobble base pair refers to a pair of nucleobases in which hydrogen bonds are formed between guanine-uracil, inosine-uracil, inosine-adenine or inosine-cytosine. As used herein, the term "complementary nucleotide sequence" does not only refers to a nucleotide sequence 100% complementary to the target nucleotide sequence but also refers to a complementary nucleotide sequence that may contain, for example, 1 to 3, 1 or 2, or one nucleotide non-complementary to the target nucleotide sequence.

As used herein, the term "stringent conditions" may be any of low stringent conditions, moderate stringent conditions or high stringent conditions. The term "low stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 32° C. The term "moderate stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 42° C., or 5×SSC, 1% SDS, 50 mM Tris-HCl (pH 7.5), 50% formamide at 42° C. The term "high stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 50° C. or 0.2×SSC, 0.1% SDS at 65° C. Under these conditions, polynucleotides with higher homology are expected to be obtained efficiently at higher temperatures, although multiple factors are involved in hybridization stringency including temperature, probe concentration, probe length, ionic strength, time, salt concentration and others, and those skilled in the art may appropriately select these factors to achieve similar stringency.

When commercially available kits are used for hybridization, for example, an Alkphos Direct Labeling and Detection System (GE Healthcare) may be used. In this case, according to the attached protocol, after cultivation with a labeled probe overnight, the membrane is washed with a primary wash buffer containing 0.1% (w/v) SDS at 55° C., thereby detecting hybridized polynucleotides. Alternatively, in producing a probe based on the entire or part of the nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1, hybridization can be detected with a DIG Nucleic Acid Detection Kit (Roche Diagnostics) when the probe is labeled with digoxigenin (DIG) using a commercially available reagent (e.g., a PCR Labeling Mix (Roche Diagnostics), etc.).

In addition to the polynucleotides described above, other polynucleotides that can be hybridized include polynucleotides having 90% or higher, 91% or higher, 92% or higher, 93% or higher, 94% or higher, 95% or higher, 96% or higher, 97% or higher, 98% or higher, 99% or higher, 99.1% or higher, 99.2% or higher, 99.3% or higher, 99.4% or higher, 99.5% or higher, 99.6% or higher, 99.7% or higher, 99.8% or higher or 99.9% or higher identity with the polynucleotide of SEQ ID NO: 1, as calculated by homology search software BLAST using the default parameters.

The identity between nucleotide sequences may be determined using algorithm BLAST (Basic Local Alignment Search Tool) by Karlin and Altschul (Proc. Natl. Acad. Sci. USA 872264-2268, 1990; Proc. Natl. Acad. Sci. USA 90: 5873, 1993). Programs called BLASTN and BLASTX based on the BLAST algorithm have been developed (Altschul S F, et al: J. Mol. Biol. 215: 403, 1990). When a nucleotide sequence is sequenced using BLASTN, the parameters are, for example, score=100 and wordlength=12. When BLAST and Gapped BLAST programs are used, the default parameters for each program are employed.

Examples of the nucleotide sequences complementary to the sequences consisting of the 31st to the 53rd, the 31st to the 54th, the 31st to the 55th, the 31st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th and the 36th to the 58th nucleotides, from the 5' end of exon 53.

TABLE 1

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NO:
31-53	5'-CCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 2
31-54	5'-TCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 3
31-55	5'-CTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 4
31-56	5'-CCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 5
31-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 6
31-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 7
32-53	5'-CCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 8
32-54	5'-TCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 9

TABLE 1 -continued

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NO:
32-55	5'-CTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 10
32-56	5'-CCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 11
32-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 12
32-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 13
33-53	5'-CCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 14
33-54	5'-TCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 15
33-55	5'-CTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 16
33-56	5'-CCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 17
33-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 18
33-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 19
34-53	5'-CCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 20
34-54	5'-TCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 21
34-55	5'-CTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 22
34-56	5'-CCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 23
34-57	5'-GCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 24
34-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 25
35-53	5'-CCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 26
35-54	5'-TCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 27
35-55	5'-CTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 28
35-56	5'-CCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 29
35-57	5'-GCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 30
35-58	5'-TGCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 31
36-53	5'-CCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 32
36-54	5'-TCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 33
36-55	5'-CTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 34
36-56	5'-CCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 35
36-57	5'-GCCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 36
36-58	5'-TGCCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 37

It is preferred that the oligomer of the present invention consists of a nucleotide sequence complementary to any one of the sequences consisting of the 32nd to the 56th, the 33rd to the 56th, the 34th to the 56th, the 35th to the 56th or the 36th to the 56th nucleotides (e.g., SEQ ID NO: 11, SEQ ID NO: 17, SEQ ID NO: 23, SEQ ID NO: 29 or SEQ ID NO: 35), from the 5' end of the 53rd exon in the human dystrophin gene.

Preferably, the oligomer of the present invention consists of a nucleotide sequence complementary to any one of the sequences consisting of the 32nd to the 56th or the 36th to the 56th nucleotides (e.g., SEQ ID NO: 11 or SEQ ID NO: 35), from the 5' end of the 53rd exon in the human dystrophin gene.

The term "cause skipping of the 53rd exon in the human dystrophin gene" is intended to mean that by binding of the oligomer of the present invention to the site corresponding to exon 53 of the transcript (e.g., pre-mRNA) of the human dystrophin gene, for example, the nucleotide sequence corresponding to the 5' end of exon 54 is spliced at the 3' side of the nucleotide sequence corresponding to the 3' end of exon 51 in DMD patients with deletion of, exon 52 when the transcript undergoes splicing, thus resulting in formation of mature mRNA which is free of codon frame shift.

Accordingly, it is not required for the oligomer of the present invention to have a nucleotide sequence 100% complementary to the target sequence, as far as it causes exon 53 skipping in the human dystrophin gene. The oligomer of the present invention may include, for example, 1 to 3, 1 or 2, or one nucleotide non-complementary to the target sequence.

Herein, the term "binding" described above is intended to mean that when the oligomer of the present invention is mixed

with the transcript of human dystrophin gene, both are hybridized under physiological conditions to form a double strand nucleic acid. The term "under physiological conditions" refers to conditions set to mimic the in vivo environment in terms of pH, salt composition and temperature. The conditions are, for example, 25 to 40° C., preferably 37° C., pH 5 to 8, preferably pH 7.4 and 150 mM of sodium chloride concentration.

Whether the skipping of exon 53 in the human dystrophin gene is caused or not can be confirmed by introducing the oligomer of the present invention into a dystrophin expression cell (e.g., human rhabdomyosarcoma cells), amplifying the region surrounding exon 53 of mRNA of the human dystrophin gene from the total RNA of the dystrophin expression cell by RT-PCR and performing nested PCR or sequence analysis on the PCR amplified product.

The skipping efficiency can be determined as follows. The mRNA for the human dystrophin gene is collected from test cells; in the mRNA, the polynucleotide level "A" of the band where exon 53 is skipped and the polynucleotide level "B" of the band where exon 53 is not skipped are measured. Using these measurement values of "A" and "B," the efficiency is calculated by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A+B) \times 100$$

The oligomer of the present invention includes, for example, an oligonucleotide, morpholino oligomer or peptide nucleic acid (PNA), having a length of 18 to 28 nucleotides. The length is preferably from 21 to 25 nucleotides and morpholino oligomers are preferred.

The oligonucleotide described above (hereinafter referred to as "the oligonucleotide of the present invention") is the oligomer of the present invention composed of nucleotides as constituent units. Such nucleotides may be any of ribonucleotides, deoxyribonucleotides and modified nucleotides.

The modified nucleotide refers to one having fully or partly modified nucleobases, sugar moieties and/or phosphate-binding regions, which constitute the ribonucleotide or deoxyribonucleotide.

The nucleobase includes, for example, adenine, guanine, hypoxanthine, cytosine, thymine, uracil, and modified bases thereof. Examples of such modified nucleobases include, but not limited to, pseudouracil, 3-methyluracil, dihydrouracil, 5-alkylcytosines (e.g., 5-methylcytosine), 5-alkyluracils (e.g., 5-ethyluracil), 5-halouracils (5-bromouracil), 6-azapyrimidine, 6-alkylpyrimidines (6-methyluracil), 2-thiouracil, 4-thiouracil, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5'-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, 1-methyladenine, 1-methylhypoxanthine, 2,2-dimethylguanine, 3-methylcytosine, 2-methyladenine, 2-methylguanine, N6-methyladenine, 7-methylguanine, 5-methoxyaminomethyl-2-thiouracil, 5-methylaminomethyluracil, 5-methylcarbonylmethyluracil, 5-methyloxyuracil, 5-methyl-2-thiouracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid, 2-thiocytosine, purine, 2,6-diaminopurine, 2-aminopurine, isoguanine, indole, imidazole, xanthine, etc.

Modification of the sugar moiety may include, for example, modifications at the 2'-position of ribose and modifications of the other positions of the sugar. The modification at the 2'-position of ribose includes replacement of the 2'-OH of ribose with OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br or I, wherein R represents an alkyl or an aryl and R' represents an alkylene.

The modification for the other positions of the sugar includes, for example, replacement of O at the 4' position of ribose or deoxyribose with S, bridging between 2' and 4' positions of the sugar, e.g., LNA (locked nucleic acid) or ENA (2'-O,4'-C-ethylene-bridged nucleic acids), but is not limited thereto.

A modification of the phosphate-binding region includes, for example, a modification of replacing phosphodiester bond with phosphorothioate bond, phosphorodithioate bond, alkyl phosphonate bond, phosphoroamidate bond or boranophosphate bond (Enya et al: Bioorganic & Medicinal Chemistry, 2008, 18, 9154-9160) (cf., e.g., Japan Domestic Re-Publications of PCT Application Nos. 2006/129594 and 2006/038608).

The alkyl is preferably a straight or branched alkyl having 1 to 6 carbon atoms. Specific examples include methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, n-pentyl, isopentyl, neopentyl, tert-pentyl, n-hexyl and isohexyl. The alkyl may optionally be substituted. Examples of such substituents are a halogen, an alkoxy, cyano and nitro. The alkyl may be substituted with 1 to 3 substituents.

The cycloalkyl is preferably a cycloalkyl having 5 to 12 carbon atoms. Specific examples include cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, cyclodecyl and cyclododecyl.

The halogen includes fluorine, chlorine, bromine and iodine.

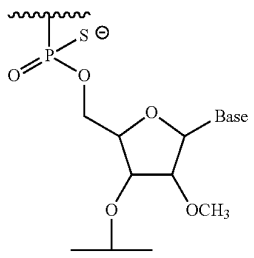
The alkoxy is a straight or branched alkoxy having 1 to 6 carbon atoms such as methoxy, ethoxy, n-propoxy, isopropoxy, n-butoxy, isobutoxy, sec-butoxy, tert-butoxy, n-pentyloxy, isopentyloxy, n-hexyloxy, isohexyloxy, etc. Among others, an alkoxy having 1 to 3 carbon atoms is preferred.

The aryl is preferably an aryl having 6 to 10 carbon atoms. Specific examples include phenyl, α-naphthyl and β-naphthyl. Among others, phenyl is preferred. The aryl may optionally be substituted. Examples of such substituents are an alkyl, a halogen, an alkoxy, cyano and nitro. The aryl may be substituted with one to three of such substituents.

The alkylene is preferably a straight or branched alkylene having 1 to 6 carbon atoms. Specific examples include methylene, ethylene, trimethylene, tetramethylene, pentamethylene, hexamethylene, 2-(ethyl) trimethylene and 1-(methyl) tetramethylene.

The acyl includes a straight or branched alkanoyl or aroyl. Examples of the alkanoyl include formyl, acetyl, 2-methylacetyl, 2,2-dimethylacetyl, propionyl, butyryl, isobutyryl, pentanoyl, 2,2-dimethylpropionyl, hexanoyl, etc. Examples of the aroyl include benzoyl, toluoyl and naphthoyl. The aroyl may optionally be substituted at substitutable positions and may be substituted with an alkyl(s).

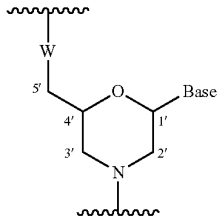
Preferably, the oligonucleotide of the present invention is the oligomer of the present invention containing a constituent unit represented by general formula below wherein the —OH group at position 2' of ribose is substituted with methoxy and the phosphate-binding region is a phosphorothioate bond:



wherein Base represents a nucleobase.

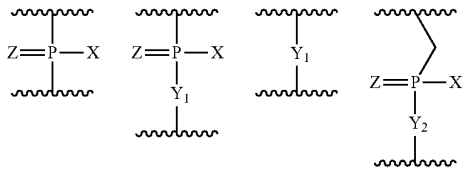
The oligonucleotide of the present invention may be easily synthesized using various automated synthesizer (e.g., AKTA oligopilot plus 10/100 (GE Healthcare)). Alternatively, the synthesis may also be entrusted to a third-party organization (e.g., Promega Inc., or Takara Co.), etc.

The morpholino oligomer of the present invention is the oligomer of the present invention comprising the constituent unit represented by general formula below:



wherein Base has the same significance as defined above, and,

W represents a group shown by any one of the following groups:



wherein X represents $—CH_2R^1$, $—O—CH_2R'$, $—S—CH_2R^1$, $—NR_2R^3$ or F;

R^1 represents H or an alkyl;

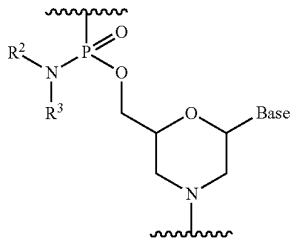
R^2 and R^3, which may be the same or different, each represents H, an alkyl, a cycloalkyl or an aryl;

Y_1 represents O, S, CH_2 or NR^1;

Y_2 represents O, S or NR^1;

Z represents O or S.

Preferably, the morpholino oligomer is an oligomer comprising a constituent unit represented by general formula below (phosphorodiamidate morpholino oligomer (hereinafter referred to as "PMO")).

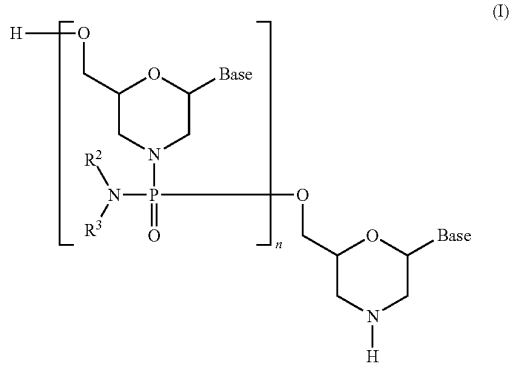


wherein Base, R^2 and R^3 have the same significance as defined above.

The morpholino oligomer may be produced in accordance with, e.g., WO 1991/009033 or WO 2009/064471. In particular, PMO can be produced by the procedure described in WO 2009/064471 or produced by the process shown below.

[Method for Producing PMO]

An embodiment of PMO is, for example, the compound represented by general formula (I) below (hereinafter PMO (I)).



(I)

wherein Base, R^2 and R^3 have the same significance as defined above; and,

n is a given integer of 1 to 99, preferably a given integer of 18 to 28.

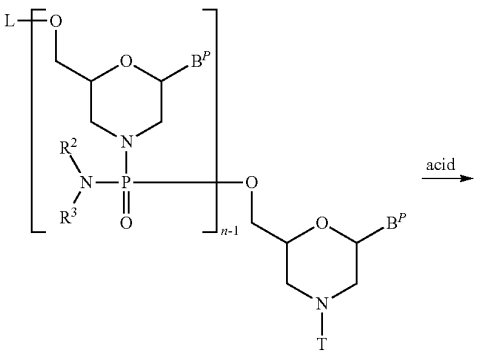
PMO (I) can be produced in accordance with a known method, for example, can be produced by performing the procedures in the following steps.

The compounds and reagents used in the steps below are not particularly limited so long as they are commonly used to prepare PMO.

Also, the following steps can all be carried out by the liquid phase method or the solid phase method (using manuals or commercially available solid phase automated synthesizers). In producing PMO by the solid phase method, it is desired to use automated synthesizers in view of simple operation procedures and accurate synthesis.

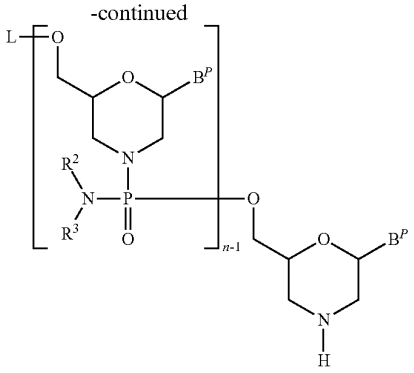
(1) Step A:

The compound represented by general formula (II) below (hereinafter referred to as Compound (II)) is reacted with an acid to prepare the compound represented by general formula (III) below (hereinafter referred to as Compound (III)):



(II)

-continued



(III)

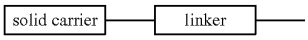
wherein n, R^2 and R^3 have the same significance as defined above;

each B^P independently represents a nucleobase which may optionally be protected;

T represents trityl, monomethoxytrityl or dimethoxytrityl; and,

L represents hydrogen, an acyl or a group represented by general formula (IV) below (hereinafter referred to as group (IV)).

(IV)



The "nucleobase" for B^P includes the same "nucleobase" as in Base, provided that the amino or hydroxy group in the nucleobase shown by B^P may be protected.

Such protective group for amino is not particularly limited so long as it is used as a protective group for nucleic acids. Specific examples include benzoyl, 4-methoxybenzoyl, acetyl, propionyl, butyryl, isobutyryl, phenylacetyl, phenoxyacetyl, 4-tert-butylphenoxyacetyl, 4-isopropylphenoxyacetyl and (dimethylamino)methylene. Specific examples of the protective group for the hydroxy group include 2-cyanoethyl, 4-nitrophenethyl, phenylsulfonylethyl, methylsulfonylethyl and trimethylsilylethyl, and phenyl, which may be substituted by 1 to 5 electron-withdrawing group at optional substitutable positions, diphenylcarbamoyl, dimethylcarbamoyl, diethylcarbamoyl, methylphenylcarbamoyl, 1-pyrrolidinylcarbamoyl, morpholinocarbamoyl, 4-(tert-butylcarboxy) benzyl, 4-[(dimethylamino)carboxyl]benzyl and 4-(phenylcarboxy)benzyl, (cf., e.g., WO 2009/064471).

The "solid carrier" is not particularly limited so long as it is a carrier usable for the solid phase reaction of nucleic acids. It is desired for the solid carrier to have the following properties: e.g., (i) it is sparingly soluble in reagents that can be used for the synthesis of morpholino nucleic acid derivatives (e.g., dichloromethane, acetonitrile, tetrazole, N-methylimidazole, pyridine, acetic anhydride, lutidine, trifluoroacetic acid); (ii) it is chemically stable to the reagents usable for the synthesis of morpholino nucleic acid derivatives; (iii) it can be chemically modified; (iv) it can be charged with desired morpholino nucleic acid derivatives; (v) it has a strength sufficient to withstand high pressure through treatments; and (vi) it has a uniform particle diameter range and distribution. Specifically, swellable polystyrene (e.g., aminomethyl polystyrene resin 1% dibenzylbenzene crosslinked (200-400 mesh) (2.4-3.0 mmol/g) (manufactured by Tokyo Chemical Industry), Aminomethylated Polystyrene Resin.HCl [dibenzylbenzene 1%, 100-200 mesh] (manufactured by Peptide Institute, Inc.)), non-swellable polystyrene (e.g., Primer Support (manufactured by GE Healthcare)), PEG chain-attached

polystyrene (e.g., NH_2—PEG resin (manufactured by Watanabe Chemical Co.), TentaGel resin), controlled pore glass (controlled pore glass; CPG) (manufactured by, e.g., CPG), oxalyl-controlled pore glass (cf., e.g., Alul et al., Nucleic Acids Research, Vol. 19, 1527 (1991)), TentaGel support-aminopolyethylene glycol-derivatized support (e.g., Wright et al., cf., Tetrahedron Letters, Vol. 34, 3373 (1993)), and a copolymer of Poros-polystyrene/divinylbenzene.

A "linker" which can be used is a known linker generally used to connect nucleic acids or morpholino nucleic acid derivatives. Examples include 3-aminopropyl, succinyl, 2,2'-diethanolsulfonyl and a long chain alkyl amino (LCAA).

This step can be performed by reacting Compound (II) with an acid.

The "acid" which can be used in this step includes, for example, trifluoroacetic acid, dichloroacetic acid and trichloroacetic acid. The acid used is appropriately in a range of, for example, 0.1 mol equivalent to 1000 mol equivalents based on 1 mol of Compound (II), preferably in a range of 1 mol equivalent to 100 mol equivalents based on 1 mol of Compound (II).

An organic amine can be used in combination with the acid described above. The organic amine is not particularly limited and includes, for example, triethylamine. The amount of the organic amine used is appropriately in a range of, e.g., 0.01 mol equivalent to 10 mol equivalents, and preferably in a range of 0.1 mol equivalent to 2 mol equivalents, based on 1 mol of the acid.

When a salt or mixture of the acid and the organic amine is used in this step, the salt or mixture includes, for example, a salt or mixture of trifluoroacetic acid and triethylamine, and more specifically, a mixture of 1 equivalent of triethylamine and 2 equivalents of trifluoroacetic acid.

The acid which can be used in this step may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, or a mixture thereof.

The reaction temperature in the reaction described above is preferably in a range of, e.g., 10° C. to 50° C., more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C.

The reaction time may vary depending upon kind of the acid used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

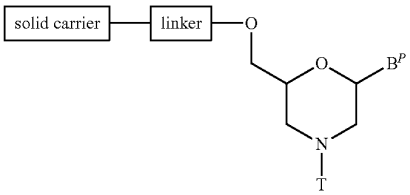
After completion of this step, a base may be added, if necessary, to neutralize the acid remained in the system. The "base" is not particularly limited and includes, for example, diisopropylamine. The base may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% (v/v) to 30% (v/v).

The solvent used in this step is not particularly limited so long as it is inert to the reaction, and includes dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, and a mixture thereof. The reaction temperature is preferably in a range of, e.g., 10° C. to 50° C., more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C.

The reaction time may vary depending upon kind of the base used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

In Compound (II), the compound of general formula (IIa) below (hereinafter Compound (IIa)), wherein n is 1 and L is a group (IV), can be produced by the following procedure.

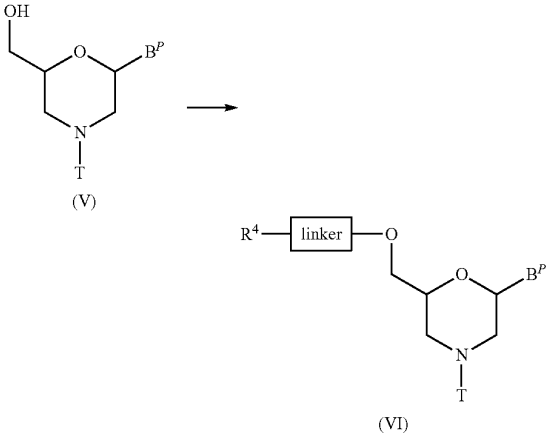
(IIa)



wherein B^P, T, linker and solid carrier have the same significance as defined above.

Step 1:

The compound represented by general formula (V) below is reacted with an acylating agent to prepare the compound represented by general formula (VI) below (hereinafter referred to as Compound (VI)).

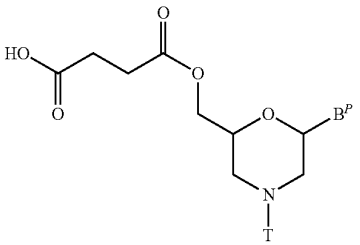


wherein B^P, T and linker have the same significance as defined above; and, R^4 represents hydroxy, a halogen or amino.

This step can be carried out by known procedures for introducing linkers, using Compound (V) as the starting material.

In particular, the compound represented by general formula (VIa) below can be produced by performing the method known as esterification, using Compound (V) and succinic anhydride.

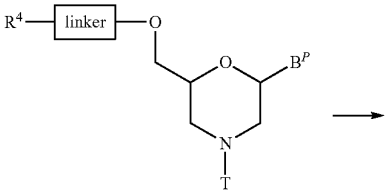
(VIa)



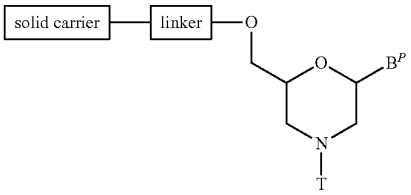
wherein B^P and T have the same significance as defined above.

Step 2:

Compound (VI) is reacted with a solid career by a condensing agent to prepare Compound (IIa).



(VI)



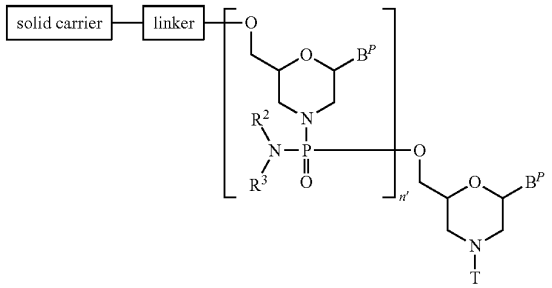
(IIa)

wherein B^P, R^4, T, linker and solid carrier have the same significance as defined above.

This step can be performed using Compound (VI) and a solid carrier in accordance with a process known as condensation reaction.

In Compound (II), the compound represented by general formula (IIa2) below wherein n is 2 to 99 and L is a group represented by general formula (IV) can be produced by using Compound (IIa) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

(IIa2)

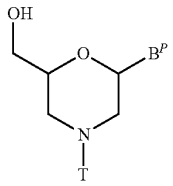


wherein B^P, R^2, R^3, T, linker and solid carrier have the same significance as defined above; and,

n' represents 1 to 98.

In Compound (II), the compound of general formula (IIb) below wherein n is 1 and L is hydrogen can be produced by the procedure described in, e.g., WO 1991/009033.

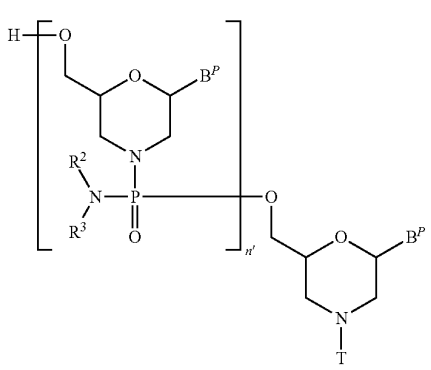
(IIb)



wherein B^P and T have the same significance as defined above.

In Compound (II), the compound represented by general formula (IIb2) below wherein n is 2 to 99 and L is hydrogen can be produced by using Compound (IIb) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

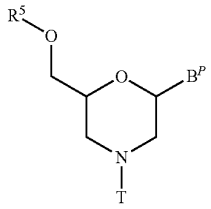
(IIb2)



wherein B^P, n', R^2, R^3 and T have the same significance as defined above.

In Compound (II), the compound represented by general formula (IIc) below wherein n is 1 and L is an acyl can be produced by performing the procedure known as acylation reaction, using Compound (IIb).

(IIc)

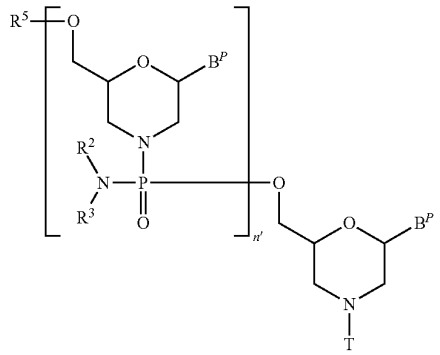


wherein B^P and T have the same significance as defined above; and,

R^5 represents an acyl.

In Compound (II), the compound represented by general formula (IIc2) below wherein n is 2 to 99 and L is an acyl can be produced by using Compound (IIc) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

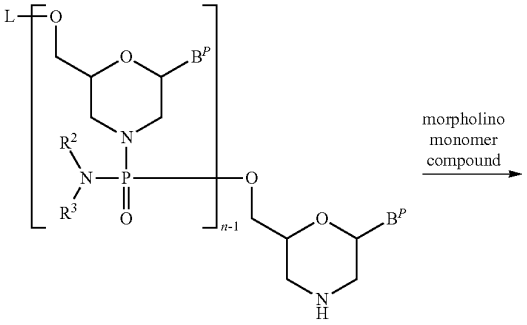
(IIc2)



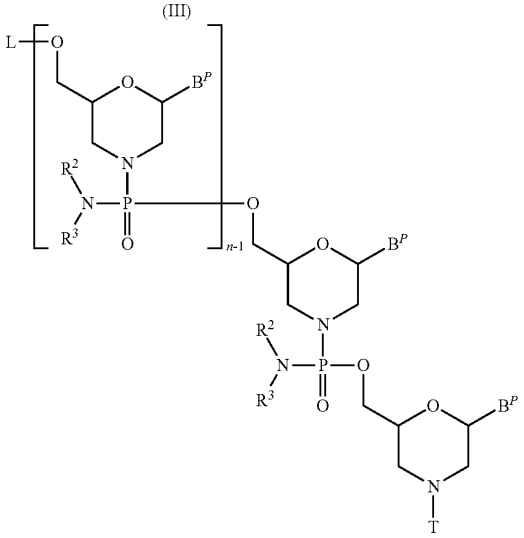
wherein B^P, n', R^2, R^3, R^5 and T have the same significance as defined above.

(2) Step B

Compound (III) is reacted with a morpholino monomer compound in the presence of a base to prepare the compound represented by general formula (VII) below (hereinafter referred to as Compound (VII)):



(III)



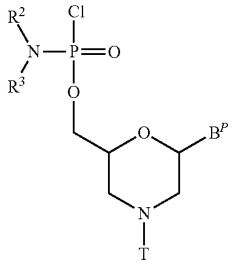
(VII)

wherein B^P, L, n, R^2, R^3 and T have the same significance as defined above.

This step can be performed by reacting Compound (III) with the morpholino monomer compound in the presence of a base.

The morpholino monomer compound includes, for example, compounds represented by general formula (VIII) below:

(VIII)



wherein B^P, R^2, R^3 and T have the same significance as defined above.

The "base" which can be used in this step includes, for example, diisopropylamine, triethylamine and N-ethylmorpholine. The amount of the base used is appropriately in a range of 1 mol equivalent to 1000 mol equivalents based on 1 mol of Compound (III), preferably, 10 mol equivalents to 100 mol equivalents based on 1 mol of Compound (III).

The morpholino monomer compound and base which can be used in this step may also be used as a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, N,N-dimethylimidazolidone, N-methylpiperidone, DMF, dichloromethane, acetonitrile, tetrahydrofuran, or a mixture thereof.

The reaction temperature is preferably in a range of e.g., 0° C. to 100° C., and more preferably, in a range of 10° C. to 50° C.

The reaction time may vary depending upon kind of the base used and reaction temperature, and is appropriately in a range of 1 minute to 48 hours in general, and preferably in a range of 30 minutes to 24 hours.

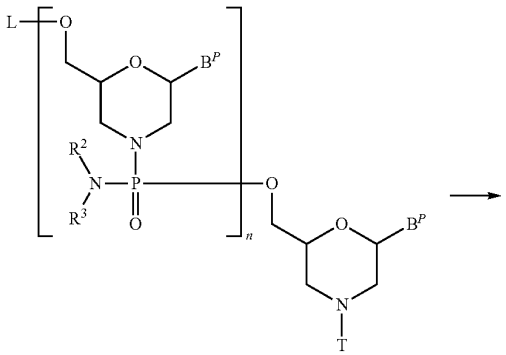
Furthermore, after completion of this step, an acylating agent can be added, if necessary. The "acylating agent" includes, for example, acetic anhydride, acetyl chloride and phenoxyacetic anhydride. The acylating agent may also be used as a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, dichloromethane, acetonitrile, an alcohol(s) (ethanol, isopropanol, trifluoroethanol, etc.), water, or a mixture thereof.

If necessary, a base such as pyridine, lutidine, collidine, triethylamine, diisopropylethylamine, N-ethylmorpholine, etc. may also be used in combination with the acylating agent. The amount of the acylating agent is appropriately in a range of 0.1 mol equivalent to 10000 mol equivalents, and preferably in a range of 1 mol equivalent to 1000 mol equivalents. The amount of the base is appropriately in a range of, e.g., 0.1 mol equivalent to 100 mol equivalents, and preferably in a range of 1 mol equivalent to 10 mol equivalents, based on 1 mol of the acylating agent.

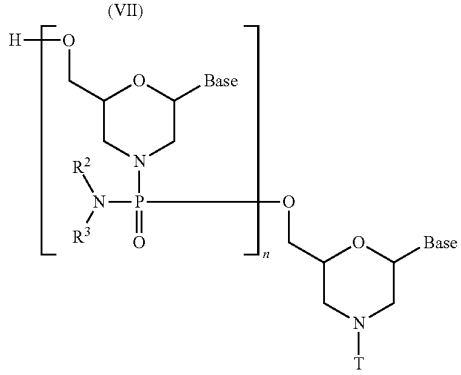
The reaction temperature in this reaction is preferably in a range of 10° C. to 50° C., more preferably, in a range of 10° C. to 50° C., much more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C. The reaction time may vary depending upon kind of the acylating agent used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

(3) Step C:

In Compound (VII) produced in Step B, the protective group is removed using a deprotecting agent to prepare the compound represented by general formula (IX).



(VII)



(IX)

wherein Base, B^P, L, n, R^2, R^3 and T have the same significance as defined above.

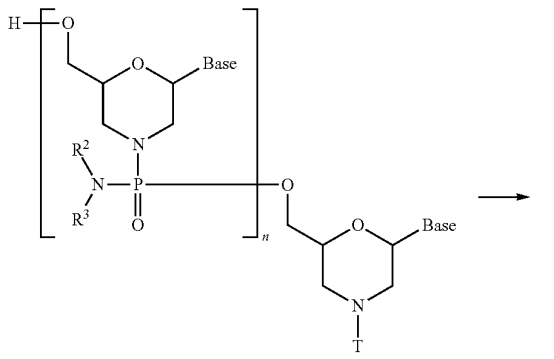
This step can be performed by reacting Compound (VII) with a deprotecting agent.

The "deprotecting agent" includes, e.g., conc. ammonia water and methylamine. The "deprotecting agent" used in this step may also be used as a dilution with, e.g., water, methanol, ethanol, isopropyl alcohol, acetonitrile, tetrahydrofuran, DMF, N,N-dimethylimidazolidone, N-methylpiperidone, or a mixture of these solvents. Among others, ethanol is preferred. The amount of the deprotecting agent used is appropriately in a range of, e.g., 1 mol equivalent to 100000 mol equivalents, and preferably in a range of 10 mol equivalents to 1000 mol equivalents, based on 1 mol of Compound (VII).

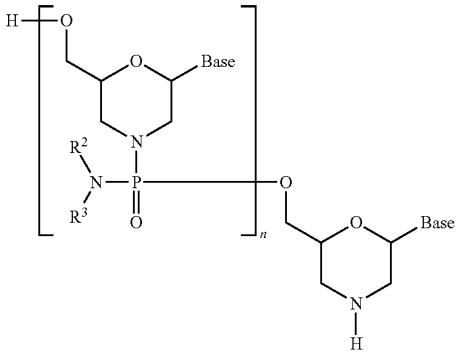
The reaction temperature is appropriately in a range of 15° C. to 75° C., preferably, in a range of 40° C. to 70° C., and more preferably, in a range of 50° C. to 60° C. The reaction time for deprotection may vary depending upon kind of Compound (VII), reaction temperature, etc., and is appropriately in a range of 10 minutes to 30 hours, preferably 30 minutes to 24 hours, and more preferably in a range of 5 hours to 20 hours.

(4) Step D:

PMO (I) is produced by reacting Compound (IX) produced in step C with an acid:



(IX)



(I)

wherein Base, n, R^2, R^3 and T have the same significance as defined above.

This step can be performed by adding an acid to Compound (IX).

The "acid" which can be used in this step includes, for example, trichloroacetic acid, dichloroacetic acid, acetic acid, phosphoric acid, hydrochloric acid, etc. The acid used is appropriately used to allow the solution to have a pH range of 0.1 to 4.0, and more preferably, in a range of pH 1.0 to 3.0. The solvent is not particularly limited so long as it is inert to the reaction, and includes, for example, acetonitrile, water, or a mixture of these solvents thereof.

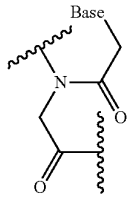
The reaction temperature is appropriately in a range of 10° C. to 50° C., preferably, in a range of 20° C. to 40° C., and more preferably, in a range of 25° C. to 35° C. The reaction time for deprotection may vary depending upon kind of Compound (IX), reaction temperature, etc., and is appropriately in a range of 0.1 minute to 5 hours, preferably 1 minute to 1 hour, and more preferably in a range of 1 minute to 30 minutes.

PMO (I) can be obtained by subjecting the reaction mixture obtained in this step to conventional means of separation and purification such as extraction, concentration, neutralization, filtration, centrifugal separation, recrystallization, reversed phase column chromatography C_8 to C_{18}, cation exchange column chromatography, anion exchange column chromatography, gel filtration column chromatography, high performance liquid chromatography, dialysis, ultrafiltration, etc., alone or in combination thereof. Thus, the desired PMO (I) can be isolated and purified (cf., e.g., WO 1991/09033).

In purification of PMO (I) using reversed phase chromatography, e.g., a solution mixture of 20 mM triethylamine/acetate buffer and acetonitrile can be used as an elution solvent.

In purification of PMO (I) using ion exchange chromatography, e.g., a solution mixture of 1 M saline solution and 10 mM sodium hydroxide aqueous solution can be used as an elution solvent.

A peptide nucleic acid is the oligomer of the present invention having a group represented by the following general formula as the constituent unit:



wherein Base has the same significance as defined above.

Peptide nucleic acids can be prepared by referring to, e.g., the following literatures.

1) P. E. Nielsen, M. Egholm, R. H. Berg, O. Buchardt, Science, 254, 1497 (1991)

2) M. Egholm, O. Buchardt, P. E. Nielsen, R. H. Berg, Jacs., 114, 1895 (1992)

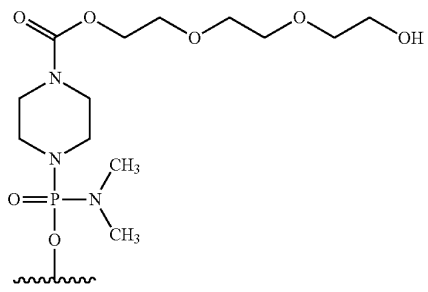
3) K. L. Dueholm, M. Egholm, C. Behrens, L. Christensen, H. F. Hansen, T. Vulpius, K. H. Petersen, R. H. Berg, P. E. Nielsen, O. Buchardt, J. Org. Chem., 59, 5767 (1994)

4) L. Christensen, R. Fitzpatrick, B. Gildea, K. H. Petersen, H. F. Hansen, T. Koch, M. Egholm, O. Buchardt, P. E. Nielsen, J. Coull, R. H. Berg, J. Pept. Sci., 1, 175 (1995)

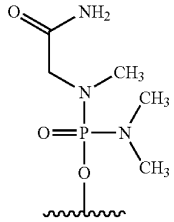
5) T. Koch, H. F. Hansen, P. Andersen, T. Larsen, H. G. Batz, K. Otteson, H. Orum, J. Pept. Res., 49, 80 (1997)

In the oligomer of the present invention, the 5' end may be any of chemical structures (1) to (3) below, and preferably is (3)-OH.

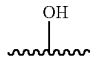
(1)



(2)



(3)



Hereinafter, the groups shown by (1), (2) and (3) above are referred to as "Group (1)," "Group (2)" and "Group (3)," respectively.

2. Pharmaceutical Composition

The oligomer of the present invention causes exon 53 skipping with a higher efficiency as compared to the prior art antisense oligomers. It is thus expected that conditions of muscular dystrophy can be relieved with high efficience by administering the pharmaceutical composition comprising the oligomer of the present invention to DMD patients. For example, when the pharmaceutical composition comprising the oligomer of the present invention is used, the same therapeutic effects can be achieved even in a smaller dose than that of the oligomers of the prior art. Accordingly, side effects can be alleviated and such is economical.

In another embodiment, the present invention provides the pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the oligomer of the present invention, a pharmaceutically acceptable salt or hydrate thereof (hereinafter referred to as "the composition of the present invention").

Examples of the pharmaceutically acceptable salt of the oligomer of the present invention contained in the composition of the present invention are alkali metal salts such as salts of sodium, potassium and lithium; alkaline earth metal salts such as salts of calcium and magnesium; metal salts such as salts of aluminum, iron, zinc, copper, nickel, cobalt, etc.; ammonium salts; organic amine salts such as salts of t-octylamine, dibenzylamine, morpholine, glucosamine, phenylglycine alkyl ester, ethylenediamine, N-methylglucamine, guanidine, diethylamine, triethylamine, dicyclohexylamine, N,N'-dibenzylethylenediamine, chloroprocaine, procaine, diethanolamine, N-benzylphenethylamine, piperazine, tetramethylammonium, tris(hydroxymethyl)aminomethane; hydrohalide salts such as salts of hydrofluorates, hydrochlorides, hydrobromides and hydroiodides; inorganic acid salts such as nitrates, perchlorates, sulfates, phosphates, etc.; lower alkane sulfonates such as methanesulfonates, trifluoromethanesulfonates and ethanesulfonates; arylsulfonates such as benzenesulfonates and p-toluenesulfonates; organic acid salts such as acetates, malates, fumarates, succinates, citrates, tartarates, oxalates, maleates, etc.; and, amino acid salts such as salts of glycine, lysine, arginine, ornithine, glutamic acid and aspartic acid. These salts may be produced by known methods. Alternatively, the oligomer of the present invention contained in the composition of the present invention may be in the form of a hydrate thereof.

Administration route for the composition of the present invention is not particularly limited so long as it is pharmaceutically acceptable route for administration, and can be chosen depending upon method of treatment. In view of easiness in delivery to muscle tissues, preferred are intravenous administration, intraarterial administration, intramuscular administration, subcutaneous administration, oral administration, tissue administration, transdermal administration, etc. Also, dosage forms which are available for the composition of the present invention are not particularly limited, and include, for example, various injections, oral agents, drips, inhalations, ointments, lotions, etc.

In administration of the oligomer of the present invention to patients with muscular dystrophy, the composition of the present invention preferably contains a carrier to promote delivery of the oligomer to muscle tissues. Such a carrier is not particularly limited as far as it is pharmaceutically acceptable, and examples include cationic carriers such as cationic liposomes, cationic polymers, etc., or carriers using viral envelope. The cationic liposomes are, for example, liposomes composed of 2-O-(2-diethylaminoethyl)carabamoyl-1,3-O-dioleoylglycerol and phospholipids as the essential constituents (hereinafter referred to as "liposome A"), Oligofectamine (registered trademark) (manufactured by Invitrogen Corp.), Lipofectin (registered trademark) (manufactured by Invitrogen Corp.), Lipofectamine (registered trademark) (manufactured by Invitrogen Corp.), Lipofectamine 2000 (registered trademark) (manufactured by Invitrogen Corp.), DMRIE-C (registered trademark) (manufactured by Invitrogen Corp.), GeneSilencer (registered trademark) (manufactured by Gene Therapy Systems), TransMessenger (registered trademark) (manufactured by QIAGEN, Inc.), TransIT TKO (registered trademark) (manufactured by Minis) and Nucleofector II (Lonza). Among others, liposome A is preferred. Examples of cationic polymers are JetSI (registered trademark) (manufactured by Qbiogene, Inc.) and Jet-PEI (registered trademark) (polyethylenimine, manufactured by Qbiogene, Inc.). An example of carriers using viral envelop is GenomeOne (registered trademark) (HVJ-E liposome, manufactured by Ishihara Sangyo). Alternatively, the medical devices described in Japanese Patent No. 2924179 and the cationic carriers described in Japanese Domestic Re-Publication PCT Nos. 2006/129594 and 2008/096690 may be used as well.

A concentration of the oligomer of the present invention contained in the composition of the present invention may vary depending on kind of the carrier, etc., and is appropriately in a range of 0.1 nM to 100 μM, preferably in a range of 1 nM to 10 μM, and more preferably in a range of 10 nM to 1 μM. A weight ratio of the oligomer of the present invention contained in the composition of the present invention and the carrier (carrier/oligomer of the present invention) may vary depending on property of the oligomer, type of the carrier, etc., and is appropriately in a range of 0.1 to 100, preferably in a range of 1 to 50, and more preferably in a range of 10 to 20.

In addition to the oligomer of the present invention and the carrier described above, pharmaceutically acceptable additives may also be optionally formulated in the composition of the present invention. Examples of such additives are emulsification aids (e.g., fatty acids having 6 to 22 carbon atoms and their pharmaceutically acceptable salts, albumin and dextran), stabilizers (e.g., cholesterol and phosphatidic acid), isotonizing agents (e.g., sodium chloride, glucose, maltose, lactose, sucrose, trehalose), and pH controlling agents (e.g., hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid, sodium hydroxide, potassium hydroxide and triethanolamine). One or more of these additives can be used. The content of the additive in the composition of the present invention is appropriately 90 wt % or less, preferably 70 wt % or less and more preferably, 50 wt % or less.

The composition of the present invention can be prepared by adding the oligomer of the present invention to a carrier dispersion and adequately stirring the mixture. Additives may be added at an appropriate step either before or after addition of the oligomer of the present invention. An aqueous solvent that can be used in adding the oligomer of the present invention is not particularly limited as far as it is pharmaceutically acceptable, and examples are injectable water or injectable distilled water, electrolyte fluid such as physiological saline, etc., and sugar fluid such as glucose fluid, maltose fluid, etc. A person skilled in the art can appropriately choose conditions for pH and temperature for such matter.

The composition of the present invention may be prepared into, e.g., a liquid form and its lyophilized preparation. The lyophilized preparation can be prepared by lyophilizing the composition of the present invention in a liquid form in a conventional manner. The lyophilization can be performed, for example, by appropriately sterilizing the composition of the present invention in a liquid form, dispensing an aliquot

into a vial container, performing preliminary freezing for 2 hours at conditions of about −40 to −20° C., performing a primary drying at 0 to 10° C. under reduced pressure, and then performing a secondary drying at about 15 to 25° C. under reduced pressure. In general, the lyophilized preparation of the composition of the present invention can be obtained by replacing the content of the vial with nitrogen gas and capping.

The lyophilized preparation of the composition of the present invention can be used in general upon reconstitution by adding an optional suitable solution (reconstitution liquid) and redissolving the preparation. Such a reconstitution liquid includes injectable water, physiological saline and other infusion fluids. A volume of the reconstitution liquid may vary depending on the intended use, etc., is not particularly limited, and is suitably 0.5 to 2-fold greater than the volume prior to lyophilization or no more than 500 mL.

It is desired to control a dose of the composition of the present invention to be administered, by taking the following factors into account: the type and dosage form of the oligomer of the present invention contained; patients' conditions including age, body weight, etc.; administration route; and the characteristics and extent of the disease. A daily dose calculated as the amount of the oligomer of the present invention is generally in a range of 0.1 mg to 10 g/human, and preferably 1 mg to 1 g/human. This numerical range may vary occasionally depending on type of the target disease, administration route and target molecule. Therefore, a dose lower than the range may be sufficient in some occasion and conversely, a dose higher than the range may be required occasionally. The composition can be administered from once to several times daily or at intervals from one day to several days.

In still another embodiment of the composition of the present invention, there is provided a pharmaceutical composition comprising a vector capable of expressing the oligonucleotide of the present invention and the carrier described above. Such an expression vector may be a vector capable of expressing a plurality of the oligonucleotides of the present invention. The composition may be formulated with pharmaceutically acceptable additives as in the case with the composition of the present invention containing the oligomer of the present invention. A concentration of the expression vector contained in the composition may vary depending upon type of the career, etc., and is appropriately in a range of 0.1 nM to 100 μM, preferably in a range of 1 nM to 10 μM, and more preferably in a range of 10 nM to 1 μM. A weight ratio of the expression vector contained in the composition and the carrier (carrier/expression vector) may vary depending on property of the expression vector, type of the carrier, etc., and is appropriately in a range of 0.1 to 100, preferably in a range of 1 to 50, and more preferably in a range of 10 to 20. The content of the carrier contained in the composition is the same as in the case with the composition of the present invention containing the oligomer of the present invention, and a method for producing the same is also the same as in the case with the composition of the present invention.

Hereinafter, the present invention will be described in more detail with reference to EXAMPLES and TEST EXAMPLES below, but is not deemed to be limited thereto.

EXAMPLES

Reference Example 1

4-{[(2S,6R)-6-(4-Benzamido-2-oxopyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin

Step 1: Production of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid

Under argon atmosphere, 22.0 g of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide and 7.04 g of 4-dimethylaminopyridine (4-DMAP) were suspended in 269 mL of dichloromethane, and 5.76 g of succinic anhydride was added to the suspension, followed by stirring at room temperature for 3 hours. To the reaction solution was added 40 mL of methanol, and the mixture was concentrated under reduced pressure. The residue was extracted using ethyl acetate and 0.5M aqueous potassium dihydrogenphosphate solution. The resulting organic layer was washed sequentially with 0.5M aqueous potassium dihydrogenphosphate solution, water and brine in the order mentioned. The resulting organic layer was dried over sodium sulfate and concentrated under reduced pressure to give 25.9 g of the product.

Step 2: Production of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin

After 23.5 g of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid was dissolved in 336 mL of pyridine (dehydrated), 4.28 g of 4-DMAP and 40.3 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were added to the solution. Then, 25.0 g of Aminomethyl Polystyrene Resin cross-linked with 1% DVB (manufactured by Tokyo Chemical Industry Co., Ltd., A1543) and 24 mL of triethylamine were added to the mixture, followed by shaking at room temperature for 4 days. After completion of the reaction, the resin was taken out by filtration. The resulting resin was washed sequentially with pyridine, methanol and dichloromethane in the order mentioned, and dried under reduced pressure. To the resulting resin were added 150 mL of tetrahydrofuran (dehydrate), 15 mL of acetic anhydride and 15 mL of 2,6-lutidine, and the mixture was shaken at room temperature for 2 hours. The resin was taken out by filtration, washed sequentially with pyridine, methanol and dichloromethane in the order mentioned, and dried under reduced pressure to give 33.7 g of the product.

The loading amount of the product was determined by measuring UV absorbance at 409 nm of the molar amount of the trityl per g resin using a known method. The loading amount of the resin was 397.4 μmol/g.

Conditions of UV measurement

Device: U-2910 (Hitachi, Ltd.)

Solvent: methanesulfonic acid

Wavelength: 265 nm

ε Value: 45000

Reference Example 2

4-Oxo-4-{[(2S,6R)-6-(6-oxo-2-[2-phenoxyacetamido]-1H-purin-9-yl)-4-tritylmorpholin-2-yl]methoxy}butanoic acid loaded onto 2-aminomethylpolystyrene resin

Step 1: Production of N^2-(phenoxyacetyl)guanosine

Guanosine, 100 g, was dried at 80° C. under reduced pressure for 24 hours. After 500 mL of pyridine (anhydrous) and 500 mL of dichloromethane (anhydrous) were added thereto, 401 mL of chlorotrimethylsilane was dropwise added to the mixture under an argon atmosphere at 0° C., followed by stirring at room temperature for 3 hours. The mixture was again ice-cooled and 66.3 g of phenoxyacetyl chloride was dropwise added thereto. Under ice cooling, the mixture was stirred for further 3 hours. To the reaction solution was added 500 mL of methanol, and the mixture was stirred at room temperature overnight. The solvent was then removed by distillation under reduced pressure. To the residue was added 500 mL of methanol, and concentration under reduced pressure was performed 3 times. To the residue was added 4 L of water, and the mixture was stirred for an hour under ice cooling. The precipitates formed were taken out by filtration, washed sequentially with water and cold methanol and then dried to give 150.2 g of the objective compound (yield: 102%) (cf.: Org. Lett. (2004), Vol. 6, No. 15, 2555-2557).

Step 2

N-{9-[(2R,6S)-6-(hydroxymethyl)-4-morpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide p-toluenesulfonate

In 480 mL of methanol was suspended 30 g of the compound obtained in Step 1, and 130 mL of 2N hydrochloric acid was added to the suspension under ice cooling. Subsequently, 56.8 g of ammonium tetraborate tetrahydrate and 16.2 g of sodium periodate were added to the mixture in the order mentioned and stirred at room temperature for 3 hours. The reaction solution was ice cooled and the insoluble matters were removed by filtration, followed by washing with 100 mL of methanol. The filtrate and washing liquid were combined and the mixture was ice cooled. To the mixture was added 11.52 g of 2-picoline borane. After stirring for 20 minutes, 54.6 g of p-toluenesulfonic acid monohydrate was slowly added to the mixture, followed by stirring at 4° C. overnight. The precipitates were taken out by filtration and washed with 500 mL of cold methanol and dried to give 17.7 g of the objective compound (yield: 43.3%).

^{1}H NMR (6, DMSO-d6): 9.9-9.2 (2H, br), 8.35 (1H, s), 7.55 (2H, m), 7.35 (2H, m), 7.10 (2H, d, J=7.82 Hz), 7.00 (3H, m), 5.95 (1H, dd, J=10.64, 2.42 Hz), 4.85 (2H, s), 4.00 (1H, m), 3.90-3.60 (2H, m), 3.50-3.20 (5H, m), 2.90 (1H, m), 2.25 (3H, s)

Step 3: Production of N-{9-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide

In 30 mL of dichloromethane was suspended 2.0 g of the compound obtained in Step 2, and 13.9 g of triethylamine and 18.3 g of trityl chloride were added to the suspension under ice cooling. The mixture was stirred at room temperature for an hour. The reaction solution was washed with saturated sodium bicarbonate aqueous solution and then with water, and dried. The organic layer was concentrated under reduced pressure. To the residue was added 40 mL of 0.2M sodium citrate buffer (pH 3)/methanol (1:4 (v/v)), and the mixture was stirred. Subsequently, 40 mL of water was added and the mixture was stirred for an hour under ice cooling. The mixture was taken out by filtration, washed with cold methanol and dried to give 1.84 g of the objective compound (yield: 82.0%).

Step 4: Production of 4-oxo-4-{[(2S,6R)-6-(6-oxo-2-[2-phenoxyacetamido]-1H-purin-9-yl)-4-tritylmorpholin-2-yl]methoxy}butanoic acid loaded onto aminomethyl polystyrene resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that N-{9-[2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of REFERENCE EXAMPLE 1.

Reference Example 3

4-{[(2S,6R)-6-(5-Methyl-2,4-dioxo-3,4-dihydropyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that 1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-5-methylpyrimidine-2,4(1H,3H)-dione was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of REFERENCE EXAMPLE 1.

Reference Example 4

1,12-Dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that 2-[2-(2-hydroxyethoxy)ethoxy]ethyl 4-tritylpiperazine-1-carboxylic acid (the compound described in WO 2009/064471) was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide.

According to the descriptions in EXAMPLES 1 to 12 and REFERENCE EXAMPLES 1 to 3 below, various types of PMO shown by PMO Nos. 1-11 and 13-16 in TABLE 2 were synthesized. The PMO synthesized was dissolved in injectable water (manufactured by Otsuka Pharmaceutical Factory, Inc.). PMO No. 12 was purchased from Gene Tools, LLC.

TABLE 2

PMO No.	Target sequence in exon 53	Note	SEQ ID NO:
1	31-55	5' end: group (3)	SEQ ID NO: 4
2	32-53	5' end: group (3)	SEQ ID NO: 8
3	32-56	5' end: group (3)	SEQ ID NO: 11
4	33-54	5' end: group (3)	SEQ ID NO: 15
5	34-58	5' end: group (3)	SEQ ID NO: 25
6	36-53	5' end: group (3)	SEQ ID NO: 32
7	36-55	5' end: group (3)	SEQ ID NO: 34
8	36-56	5' end: group (3)	SEQ ID NO: 35
9	36-57	5' end: group (3)	SEQ ID NO: 36

TABLE 2-continued

PMO No.	Target sequence in exon 53	Note	SEQ ID NO:
10	33-57	5' end: group (3)	SEQ ID NO: 18
11	39-69	Sequence corresponding to H53A(+39 + 69) (cf. Table 1) in Non-Patent Document 3, 5' end: group (3)	SEQ ID NO: 38
12	30-59	Sequence corresponding to h53A30/1 (cf. Table 1) in Non-Patent Document 5, 5' end: group (2)	SEQ ID NO: 39
13	32-56	5' end: group (1)	SEQ ID NO: 11
14	36-56	5' end: group (1)	SEQ ID NO: 35
15	30-59	Sequence corresponding to h53A30/1 (cf. Table 1) in Non-Patent Document 5 5' end: group (3)	SEQ ID NO: 39
16	23-47	Sequence corresponding to SEQ ID NO: 429 described in Patent Document 4, 5' end: group (3)	SEQ ID NO: 47

Example 1

PMO No. 8

4-{[(2S,6R)-6-(4-Benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid, loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 1), 2 g (800 μmol) was transferred to a reaction vessel, and 30 mL of dichloromethane was added thereto. The mixture was allowed to stand for 30 minutes. After the mixture was further washed twice with 30 mL of dichloromethane, the following synthesis cycle was started. The desired morpholino monomer compound was added in each cycle to give the nucleotide sequence of the title compound.

TABLE 3

Step	Reagent	Volume (mL)	Time (min)
1	deblocking solution	30	2.0
2	deblocking solution	30	2.0
3	deblocking solution	30	2.0
4	deblocking solution	30	2.0
5	deblocking solution	30	2.0
6	deblocking solution	30	2.0
7	neutralizing solution	30	1.5
8	neutralizing solution	30	1.5
9	neutralizing solution	30	1.5
10	neutralizing solution	30	1.5
11	neutralizing solution	30	1.5
12	neutralizing solution	30	1.5
13	dichloromethane	30	0.5
14	dichloromethane	30	0.5
15	dichloromethane	30	0.5
16	coupling solution B	20	0.5
17	coupling solution A	6-11	90.0
18	dichloromethane	30	0.5
19	dichloromethane	30	0.5
20	dichloromethane	30	0.5
21	capping solution	30	3.0
22	capping solution	30	3.0
23	dichloromethane	30	0.5
24	dichloromethane	30	0.5
25	dichloromethane	30	0.5

The deblocking solution used was a solution obtained by dissolving a mixture of trifluoroacetic acid (2 equivalents) and triethylamine (1 equivalent) in a dichloromethane solution containing 1% (v/v) ethanol and 10% (v/v) 2,2,2-trifluoroethanol to be 3% (w/v). The neutralizing solution used was a solution obtained by dissolving N,N-diisopropylethylamine in a dichloromethane solution containing 25% (v/v) 2-propanol to be 5% (v/v). The coupling solution A used was a solution obtained by dissolving the morpholino monomer compound in 1,3-dimethyl-2-imidazolidinone containing 10% (v/v) N,N-diisopropylethylamine to be 0.15M. The coupling solution B used was a solution obtained by dissolving N,N-diisopropylethylamine in 1,3-dimethyl-2-imidazolidinone to be 10% (v/v). The capping solution used was a solution obtained by dissolving 20% (v/v) acetic anhydride and 30% (v/v) 2,6-lutidine in dichloromethane.

The aminomethyl polystyrene resin loaded with the PMO synthesized above was recovered from the reaction vessel and dried at room temperature for at least 2 hours under reduced pressure. The dried PMO loaded onto aminomethyl polystyrene resin was charged in a reaction vessel, and 200 mL of 28% ammonia water-ethanol (1/4) was added thereto. The mixture was stirred at 55° C. for 15 hours. The aminomethyl polystyrene resin was separated by filtration and washed with 50 mL of water-ethanol (1/4). The resulting filtrate was concentrated under reduced pressure. The resulting residue was dissolved in 100 mL of a solvent mixture of 20 mM acetic acid-triethylamine buffer (TEAA buffer) and acetonitrile (4/1) and filtered through a membrane filter. The filtrate obtained was purified by reversed phase HPLC. The conditions used are as follows.

TABLE 4

Column	XTerra MS18 (Waters, ϕ50x 100 mm, 1CV = 200 mL)
Flow rate	60 mL/min
Column temperature	room temperature
Solution A	20 mM TEAA buffer
Solution B	CH_3CN
Gradient	(B) conc. 20→50%/9CV

Each fraction was analyzed and the product was recovered in 100 mL of acetonitrile-water (1/1), to which 200 mL of ethanol was added. The mixture was concentrated under reduced pressure. Further drying under reduced pressure gave a white solid. To the resulting solid was added 300 mL of 10 mM phosphoric acid aqueous solution to suspend the solid. To the suspension was added 10 mL of 2M phosphoric acid aqueous solution, and the mixture was stirred for 15 minutes. Furthermore, 15 mL of 2M sodium hydrate aqueous solution was added for neutralization. Then, 15 mL of 2M sodium hydroxide aqueous solution was added to make the mixture alkaline, followed by filtration through a membrane filter (0.45 μm). The mixture was thoroughly washed with 100 mL of 10 mM sodium hydroxide aqueous solution to give the product as an aqueous solution.

The resulting aqueous solution containing the product was purified by an anionic exchange resin column. The conditions used are as follows.

TABLE 5

Column	Source 30Q (GE Healthcare, ϕ40x 150 mm, 1CV = 200 mL)
Flow rate	80 mL/min
Column temp.	room temperature
Solution A	10 mM sodium hydroxide aqueous solution
Solution B	10 mM sodium hydroxide aqueous solution, 1M sodium chloride aqueous solution
Gradient	(B) conc. 5→35%/15CV

Each fraction was analyzed (on HPLC) and the product was obtained as an aqueous solution. To the resulting aqueous solution was added 225 mL of 0.1M phosphate buffer (pH 6.0) for neutralization. The mixture was filtered through a membrane filter (0.45 μm). Next, ultrafiltration was performed under the conditions described below.

TABLE 6

Filter	PELLICON2 MINI FILTER PLBC 3K Regenerated Cellulose, Screen Type C
Size	0.1 m^2

The filtrate was concentrated to give approximately 250 mL of an aqueous solution. The resulting aqueous solution was filtered through a membrane filter (0.45 μm). The aqueous solution obtained was freeze-dried to give 1.5 g of the objective compound as a white cotton-like solid.

ESI-TOF-MS Calcd.: 6924.82.

Found: 6923.54.

Example 2

PMO. No. 1

The title compound was produced in accordance with the procedure of EXAMPLE 1.

MALDI-TOF-MS Calcd.: 8291.96.

Found: 8296.24.

Example 3

PMO. No. 2

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 7310.13.

Found: 7309.23.

Example 4

PMO. No. 3

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 8270.94.

Found: 8270.55.

Example 5

PMO. No. 4

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl)methoxy)-4-oxobutanoic acid (REFERENCE EXAMPLE 3) loaded onto aminomethyl polystyrene resin was used as the starting material.

ESI-TOF-MS Calcd.: 7310.13.

Found: 7310.17.

Example 6

PMO. No. 5

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl)methoxy)-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 3) was used as the starting material.

ESI-TOF-MS Calcd.: 8270.94.

Found: 8270.20.

Example 7

PMO. No. 6

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 5964.01.

Found: 5963.68.

Example 8

PMO. No. 7

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 6609.55.

Found: 6608.85.

Example 9

PMO. No. 9

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-oxo-4-(((2S,6R)-6-(6-oxo-2-(2-phenoxyacetamido)-1H-purin-9(6H)-yl)-4-tritylmorpholin-2-yl)methoxy)butanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 2) was used as the starting material.

ESI-TOF-MS Calcd.: 7280.11.

Found: 7279.42.

Example 10

PMO. No. 10

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-oxo-4-(((2S,6R)-6-(6-oxo-2-(2-phenoxyacetamido)-1H-purin-9(6H)-yl)-4-tritylmorpholin-2-yl)methoxy)butanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 2) was used as the starting material.

ESI-TOF-MS Calcd.: 8295.95.

Found: 8295.91.

Example 11

PMO. No. 13

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 1,12-dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 4) was used as the starting material.

ESI-TOF-MS Calcd.: 7276.15.

Found: 7276.69.

Example 12

PMO. No. 14

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 1,12-dioxo-1-(4-tri-

tylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 4) was used as the starting material.

ESI-TOF-MS Calcd.: 8622.27.

Found: 8622.29.

Comparative Example 1

PMO. No. 11

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 10274.63.

Found: 10273.71.

Comparative Example 2

PMO. No. 15

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 9941.33.

Found: 9940.77.

Comparative Example 3

PMO. No. 16

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 8238.94.

Found: 8238.69.

Test Example 1

In Vitro Assay

Using an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza), 10 μM of the oligomers PMO Nos. 1 to 8 of the present invention and the antisense oligomer PMO No. 11 were transfected with 4×10^5 of RD cells (human rhabdomyosarcoma cell line). The Program T-030 was used.

After transfection, the cells were cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen) under conditions of 37° C. and 5% CO_2. The cells were washed twice with PBS (manufactured by Nissui, hereinafter the same) and 500 μl of ISOGEN (manufactured by Nippon Gene) was added to the cells. After the cells were allowed to stand at room temperature for a few minutes to lyse the cells, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a Titan One Tube RT-PCR Kit (manufactured by Roche). A reaction solution was prepared in accordance with the protocol attached to the kit. A PTC-100 (manufactured by MJ Research) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

94° C., 2 mins: thermal denaturation

[94° C., 10 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

(SEQ ID NO: 40)
Forward primer: 5'-AGGATTTGGAACAGAGGCGTC-3'

(SEQ ID NO: 41)
Reverse primer: 5'-GTCTGCCACTGGCGGAGGTC-3'

Next, a nested PCR was performed with the product amplified by RT-PCR above using a Taq DNA Polymerase (manufactured by Roche). The PCR program used is as follows.

94° C., 2 mins: thermal denaturation

[94° C., 15 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above are given below.

(SEQ ID NO: 42)
Forward primer: 5'-CATCAAGCAGAAGGCAACAA-3'

(SEQ ID NO: 43)
Reverse primer: 5'-GAAGTTTCAGGGCCAAGTCA-3'

The reaction product, 1 of the nested PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)}=A/(A+B)\times100$$

Experimental Results

The results are shown in FIG. **1**. This experiment revealed that the oligomers PMO Nos. 1 to 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the antisense oligomer PMO No. 11. In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than four times higher exon skipping efficiency than that of the antisense oligomer PMO No. 11.

Test Example 2

In Vitro Assay Using Human Fibroblasts

Human myoD gene (SEQ ID NO: 44) was introduced into TIG-119 cells (human normal tissue-derived fibroblasts, National Institute of Biomedical Innovation) or 5017 cells (human DMD patient-derived fibroblasts, Coriell Institute for Medical Research) using a ZsGreen1 coexpression retroviral vector.

After incubation for 4 to 5 days, ZsGreen-positive MyoD-transformed fibroblasts were collected by FACS and plated at $5\times10^4/cm^2$ into a 12-well plate. As a growth medium, there was used 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM.F-12) (Invitrogen Corp.) containing 10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

The medium was replaced 24 hours later by differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). The medium was exchanged every 2 to 3 days and incubation was continued for 12 to 14 days to differentiate into myotubes.

Subsequently, the differentiation medium was replaced by a differentiation medium containing 6 μM Endo-Porter (Gene Tools), and the morpholino oligomer was added thereto in a final concentration of 10 μM. After incubation for 48 hours, total RNA was extracted from the cells using a TRIzol (manufactured by Invitrogen Corp.). RT-PCR was performed with 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. A reaction solution was prepared in accordance with the protocol attached to the kit. An iCycler (manufactured by Bio-Rad) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 1 mins; 60° C., 1 mins; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 7 mins: final extension

The primers used were hEX51F and hEX55R.

(SEQ ID NO: 45)
hEX51F: 5'-CGGGCTTGGACAGAACTTAC-3'

(SEQ ID NO: 46)
hEx55R: 5'-TCCTTACGGGTAGCATCCTG-3'

The reaction product of RT-PCR above was separated by 2% agarose gel electrophoresis and gel images were captured with a GeneFlash (Syngene). The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured using an Image J (manufactured by National Institutes of Health). Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation.

$$\text{Skipping efficiency (\%)} = A/(A+B) \times 100$$

Experimental Results

The results are shown in FIGS. **2** and **3**. This experiment revealed that in TIG-119 cells, the oligomers PMO Nos. 3, 8 and 9 of the present invention (FIG. **2**) all caused exon 53 skipping with a higher efficiency than the antisense oligomer PMO No. 12 (FIG. **2**). In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than twice higher exon skipping efficiency than that of the antisense oligomer PMO No. 12 (FIG. **2**).

Furthermore, this experiment revealed that the oligomers PMO Nos. 3 and 8 to 10 of the present invention (FIG. **3**) all caused exon 53 skipping with a higher efficiency than the antisense oligomer PMO No. 12 (FIG. **3**). In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than seven times higher exon skipping efficiency than that of the antisense oligomer PMO No. 12 (FIG. **3**).

Test Example 3

In Vitro Assay Using Human Fibroblasts

The skin fibroblast cell line (fibroblasts from human DMD patient (exons 45-52 or exons 48-52)) was established by biopsy from the medial left upper arm of DMD patient with deletion of exons 45-52 or DMD patient with deletion of exons 48-52. Human myoD gene (SEQ ID NO: 44) was introduced into the fibroblast cells using a ZsGreen1 coexpression retroviral vector.

After incubation for 4 to 5 days, ZsGreen-positive MyoD-transformed fibroblasts were collected by FACS and plated at $5\times10^4/cm^2$ into a 12-well plate. As a growth medium, there was used 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen Corp.) containing 10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

The medium was replaced 24 hours later by a differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). The medium was exchanged every 2 to 3 days and incubation was continued for 12, 14 or 20 days to differentiate into myotubes.

Subsequently, the differentiation medium was replaced by a differentiation medium containing 6 μM Endo-Porter (Gene Tools), and a morpholino oligomer was added thereto at a final concentration of 10 μM. After incubation for 48 hours, total RNA was extracted from the cells using a TRIzol (manufactured by Invitrogen Corp.). RT-PCR was performed with 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. A reaction solution was prepared in accordance with the protocol attached to the kit. An iCycler (manufactured by Bio-Rad) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 1 mins; 60° C., 1 mins; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 7 mins: final extension

The primers used were hEx44F and h55R.

(SEQ ID NO: 48)
hEx44F: 5'-TGTTGAGAAATGGCGGCGT-3'

(SEQ ID NO: 46)
hEx55R: 5'-TCCTTACGGGTAGCATCCTG-3'

The reaction product of RT-PCR above was separated by 2% agarose gel electrophoresis and gel images were captured with a GeneFlash (Syngene). The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured using an Image J (manufactured by National Institutes of Health). Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation.

$$\text{Skipping efficiency (\%)} = A/(A+B) \times 100$$

Experimental Results

The results are shown in FIGS. **4** and **5**. This experiment revealed that the oligomers PMO Nos. 3 and 8 of the present invention caused exon 53 skipping with an efficiency as high as more than 80% in the cells from DMD patient with deletion of exons 45-52 (FIG. **4**) or deletion of exons 48-52 (FIG. **5**). Also, the oligomers PMO Nos. 3 and 8 of the present invention were found to cause exon 53 skipping with a higher efficiency than that of the antisense oligomer PMO No. 15 in the cells from DMD patient with deletion of exons 45-52 (FIG. **4**).

Test Example 4

Western Blotting

The oligomer PMO No. 8 of the present invention was added to the cells at a concentration of 10 μM, and proteins were extracted from the cells after 72 hours using a RIPA buffer (manufactured by Thermo Fisher Scientific) containing Complete Mini (manufactured by Roche Applied Science) and quantified using a BCA protein assay kit (manufactured by Thermo Fisher Scientific). The proteins were

electrophoresed in NuPAGE Novex Tris-Acetate Gel 3-8% (manufactured by Invitrogen) at 150V for 75 minutes and transferred onto a PVDF membrane (manufactured by Millipore) using a semi-dry blotter. The PVDF membrane was blocked with a 5% ECL Blocking agent (manufactured by GE Healthcare) and the membrane was then incubated in a solution of anti-dystrophin antibody (manufactured by NCL-Dysl, Novocastra). After further incubation in a solution of peroxidase-conjugated goat-antimouse IgG (Model No. 170-6516, Bio-Rad), the membrane was stained with ECL Plus Western blotting system (manufactured by GE Healthcare).

Immunostaining

The oligomer PMO No. 3 or 8 of the present invention was added to the cells. The cells after 72 hours were fixed in 3% paraformaldehyde for 10 minutes, followed by incubation in 10% Triton-X for 10 minutes. After blocking in 10% goat serum-containing PBS, the membrane was incubated in a solution of anti-dystrophin antibody (NCL-Dysl, Novocastra). The membrane was further incubated in a solution of anti-mouse IgG antibody (manufactured by Invitrogen). The membrane was mounted with Pro Long Gold Antifade reagent (manufactured by Invitrogen) and observed with a fluorescence microscope.

Experimental Results

The results are shown in FIGS. **6** and **7**. In this experiment it was confirmed by western blotting (FIG. **6**) and immunostaining (FIG. **7**) that the oligomers PMO Nos. 3 and 8 of the present invention induced expression of the dystrophin protein.

Test Example 5

In Vitro Assay Using Human Fibroblasts

The experiment was performed as in TEST EXAMPLE 3.

Experimental Results

The results are shown in FIG. **8**. This experiment revealed that in the cells from DMD patients with deletion of exons 45-52, the oligomers PMO Nos. 3 to 8 of the present invention caused exon 53 skipping with a higher efficiency than the oligomers PMO Nos. 13 and 14 of the present invention (FIG. **8**).

Test Example 6

In Vitro Assay

Experiments were performed using the antisense oligomers of 2'-O-methoxy-phosphorothioates (2'-OMe-S-RNA) shown by SEQ ID NO: 49 to SEQ ID NO: 123. Various antisense oligomers used for the assay were purchased from Japan Bio Services. The sequences of various antisense oligomers are given below.

TABLE 7

oligomer Antisense	Nucleotide sequence	SEQ ID NO:
H53_39-69	CAUUCAACUGUUGCCUCCGGUUCUGAAGGUG	49
H53_1-25	UCCCACUGAUUCUGAAUUCUUUCAA	50
H53_6-30	CUUCAUCCCACUGAUUCUGAAUUCU	51
H53_11-35	UUGUACUUCAUCCCACUGAUUCUGA	52
H53_16-40	UGUUCUUGUACUUCAUCCCACUGAU	53

TABLE 7 -continued

oligomer Antisense	Nucleotide sequence	SEQ ID NO:
H53_21-45	GAAGGUGUUCUUGUACUUCAUCCCA	54
H53_26-50	GUUCUGAAGGUGUUCUUGUACUUCA	55
H53_31-55	CUCCGGUUCUGAAGGUGUUCUUGUA	56
H53_36-60	GUUGCCUCCGGUUCUGAAGGUGUUC	57
H53_41-65	CAACUGUUGCCUCCGGUUCUGAAGG	58
H53_46-70	UCAUUCAACUGUUGCCUCCGGUUCU	59
H53_51-75	ACAUUUCAUUCAACUGUUGCCUCCG	60
H53_56-80	CUUUAACAUUUCAUUCAACUGUUGC	61
H53_61-85	GAAUCCUUUAACAUUUCAUUCAACU	62
H53_66-90	GUGUUGAAUCCUUUAACAUUUCAUU	63
H53_71-95	CCAUUGUGUUGAAUCCUUUAACAUU	64
H53_76-100	UCCAGCCAUUGUGUUGAAUCCUUUA	65
H53_81-105	UAGCUUCCAGCCAUUGUGUUGAAUC	66
H53_86-110	UUCCUUAGCUUCCAGCCAUUGUGUU	67
H53_91-115	GCUUCUUCCUUAGCUUCCAGCCAUU	68
H53_96-120	GCUCAGCUUCUUCCUUAGCUUCCAG	69
H53_101-125	GACCUGCUCAGCUUCUUCCUUAGCU	70
H53_106-130	CCUAAGACCUGCUCAGCUUCUUCCU	71
H53_111-135	CCUGUCCUAAGACCUGCUCAGCUUC	72
H53_116-140	UCUGGCCUGUCCUAAGACCUGCUCA	73
H53_121-145	UUGGCUCUGGCCUGUCCUAAGACCU	74
H53_126-150	CAAGCUUGGCUCUGGCCUGUCCUAA	75
H53_131-155	UGACUCAAGCUUGGCUCUGGCCUGU	76
H53_136-160	UUCCAUGACUCAAGCUUGGCUCUGG	77
H53_141-165	CCUCCUUCCAUGACUCAAGCUUGGC	78
H53_146-170	GGGACCCUCCUUCCAUGACUCAAGC	79
H53_151-175	GUAUAGGGACCCUCCUUCCAUGACU	80
H53_156-180	CUACUGUAUAGGGACCCUCCUUCCA	81
H53_161-185	UGCAUCUACUGUAUAGGGACCCUCC	82
H53_166-190	UGGAUUGCAUCUACUGUAUAGGGAC	83
H53_171-195	UCUUUUGGAUUGCAUCUACUGUAUA	84
H53_176-200	GAUUUUCUUUUGGAUUGCAUCUACU	85
H53_181-205	UCUGUGAUUUUCUUUUGGAUUGCAU	86
H53_186-210	UGGUUUCUGUGAUUUUCUUUUGGAU	87
H53_84-108	CCUUAGCUUCCAGCCAUUGUGUUGA	88
H53_88-112	UCUUCCUUAGCUUCCAGCCAUUGUG	89
H53_119-143	GGCUCUGGCCUGUCCUAAGACCUGC	90
H53_124-148	AGCUUGGCUCUGGCCUGUCCUAAGA	91
H53_128-152	CUCAAGCUUGGCUCUGGCCUGUCCU	92

TABLE 7 -continued

oligomer Antisense	Nucleotide sequence	SEQ ID NO:
H53_144-168	GACCCUCCUUCCAUGACUCAAGCUU	93
H53_149-173	AUAGGGACCCUCCUUCCAUGACUCA	94
H53_153-177	CUGUAUAGGGACCCUCCUUCCAUGA	95
H53_179-203	UGUGAUUUUCUUUUGGAUUGCAUCU	96
H53_184-208	GUUUCUGUGAUUUUCUUUUGGAUUG	97
H53_188-212	CUUGGUUUCUGUGAUUUUCUUUUGG	98
H53_29-53	CCGGUUCUGAAGGUGUUCUUGUACU	99
H53_30-54	UCCGGUUCUGAAGGUGUUCUUGUAC	100
H53_32-56	CCUCCGGUUCUGAAGGUGUUCUUGU	101
H53_33-57	GCCUCCGGUUCUGAAGGUGUUCUUG	102
H53_34-58	UGCCUCCGGUUCUGAAGGUGUUCUU	103
H53_35-59	UUGCCUCCGGUUCUGAAGGUGUUCU	104
H53_37-61	UGUUGCCUCCGGUUCUGAAGGUGUU	105
H53_38-62	CUGUUGCCUCCGGUUCUGAAGGUGU	106
H53_39-63	ACUGUUGCCUCCGGUUCUGAAGGUG	107
H53_40-64	AACUGUUGCCUCCGGUUCUGAAGGU	108
H53_32-61	UGUUGCCUCCGGUUCUGAAGGUGUUCUUGU	109
H53_32-51	GGUUCUGAAGGUGUUCUUGU	110
H53_35-54	UCCGGUUCUGAAGGUGUUCU	111
H53_37-56	CCUCCGGUUCUGAAGGUGUU	112
H53_40-59	UUGCCUCCGGUUCUGAAGGU	113
H53_42-61	UGUUGCCUCCGGUUCUGAAG	114
H53_32-49	UUCUGAAGGUGUUCUUGU	115
H53_35-52	CGGUUCUGAAGGUGUUCU	116
H53_38-55	CUCCGGUUCUGAAGGUGU	117
H53_41-58	UGCCUCCGGUUCUGAAGG	118
H53_44-61	UGUUGCCUCCGGUUCUGA	119
H53_35-49	UUCUGAAGGUGUUCU	120
H53_40-54	UCCGGUUCUGAAGGU	121
H53_45-59	UUGCCUCCGGUUCUG	122
H53_45-62	CUGUUGCCUCCGGUUCUG	123

RD cells (human rhabdomyosarcoma cell line) were plated at 3×10^5 in a 6-well plate and cultured in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, Inc., hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen Corp.) under conditions of 37° C. and 5% CO_2 overnight. Complexes of various antisense oligomers (Japan Bio Services) (1 μM) for exon 53 skipping and Lipofectamine 2000 (manufactured by Invitrogen Corp.) were prepared and 200 μl was added to RD cells where 1.8 mL of the medium was exchanged, to reach the final concentration of 100 nM.

After completion of the addition, the cells were cultured overnight. The cells were washed twice with PBS (manufactured by Nissui, hereafter the same) and then 500 μl of ISOGEN (manufactured by Nippon Gene) were added to the cells. After the cells were allowed to stand at room temperature for a few minutes for cell lysis, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a Titan One Tube RT-PCR Kit (manufactured by Roche). A reaction solution was prepared in accordance with the protocol attached to the kit. A PTC-100 (manufactured by MJ Research) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

94° C., 2 mins: thermal denaturation

[94° C., 10 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

(SEQ ID NO: 42)
Forward primer: 5'-CATCAAGCAGAAGGCAACAA-3'

(SEQ ID NO: 43)
Reverse primer: 5'-GAAGTTTCAGGGCCAAGTCA-3'

Subsequently, a nested PCR was performed with the amplified product of RT-PCR above using a Taq DNA Polymerase (manufactured by Roche). The PCR program used is as follows.

94° C., 2 mins: thermal denaturation

[94° C., 15 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above are given below.

(SEQ ID NO: 40)
Forward primer: 5'-AGGATTTGGAACAGAGGCGTC-3'

(SEQ ID NO: 41)
Reverse primer: 5'-GTCTGCCACTGGCGGAGGTC-3'

The reaction product, 1 μl, of the nested PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A+B)\times 100$$

Experimental Results

The results are shown in FIGS. **9** to **17**. These experiments revealed that, when the antisense oligomers were designed at exons 31-61 from the 5' end of exon 53 in the human dystrophin gene, exon 53 skipping could be caused with a high efficiency.

Test Example 7

Using an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza), 0.3 to 30 μM of the antisense oligomers

were transfected with 3.5×10^5 of RD cells (human rhabdomyosarcoma cell line). The Program T-030 was used.

After the transfection, the cells were cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, Inc., hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen Corp.) under conditions of 37° C. and 5% CO_2. The cells were washed twice with PBS (manufactured by Nissui, hereinafter the same) and 500 μl of ISOGEN (manufactured by Nippon Gene) was then added to the cells. After the cells were allowed to stand at room temperature for a few minutes to lyse the cells, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit (manufactured by Qiagen, Inc.). A reaction solution was prepared in accordance with the protocol attached to the kit. The thermal cycler used was a PTC-100 (manufactured by MJ Research). The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 30 seconds; 60° C., 30 seconds; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 10 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

(SEQ ID NO: 42)
Forward primer: 5'-CATCAAGCAGAAGGCAACAA-3'

(SEQ ID NO: 43)
Reverse primer: 5'-GAAGTTTCAGGGCCAAGTCA-3'

The reaction product, 1 μl, of the PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)}=A/(A+B)\times100$$

Experimental Results

The results are shown in FIGS. **18** and **19**. These experiments revealed that the oligomer PMO No. 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the antisense oligomers PMO Nos. 15 and 16 (FIG. **18**). It was also revealed that the oligomers PMO Nos. 3 and 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the oligomers PMO Nos. 13 and 14 of the present invention (FIG. **19**). These results showed that the sequences with —OH group at the 5' end provide a higher skipping efficiency even in the same sequences.

INDUSTRIAL APPLICABILITY

Experimental results in TEST EXAMPLES demonstrate that the oligomers of the present invention (PMO Nos. 1 to 10) all caused exon 53 skipping with a markedly high efficiency under all cell environments, as compared to the oligomers (PMO Nos. 11, 12, 15 and 16) in accordance with the prior art. The 5017 cells used in TEST EXAMPLE 2 are the cells isolated from DMD patients, and the fibroblasts used in TEST EXAMPLES 3 and 5 are exon 53 skipping target cells from DMD patients. Particularly in TEST EXAMPLES 3 and 5, the oligomers of the present invention show the exon 53 skipping efficiency of 90% or higher in the cells from DMD patients that are the target for exon 53 skipping. Consequently, the oligomers of the present invention can induce exon 53 skipping with a high efficiency, when DMD patients are administered.

Therefore, the oligomers of the present invention are extremely useful for the treatment of DMD.

Sequence Listing Free Text

SEQ ID NO: 2: synthetic nucleic acid
SEQ ID NO: 3: synthetic nucleic acid
SEQ ID NO: 4: synthetic nucleic acid
SEQ ID NO: 5: synthetic nucleic acid
SEQ ID NO: 6: synthetic nucleic acid
SEQ ID NO: 7: synthetic nucleic acid
SEQ ID NO: 8: synthetic nucleic acid
SEQ ID NO: 9: synthetic nucleic acid
SEQ ID NO: 10: synthetic nucleic acid
SEQ ID NO: 11: synthetic nucleic acid
SEQ ID NO: 12: synthetic nucleic acid
SEQ ID NO: 13: synthetic nucleic acid
SEQ ID NO: 14: synthetic nucleic acid
SEQ ID NO: 15: synthetic nucleic acid
SEQ ID NO: 16: synthetic nucleic acid
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SEQ ID NO: 113: synthetic nucleic acid
SEQ ID NO: 114: synthetic nucleic acid
SEQ ID NO: 115: synthetic nucleic acid
SEQ ID NO: 116: synthetic nucleic acid
SEQ ID NO: 117: synthetic nucleic acid
SEQ ID NO: 118: synthetic nucleic acid
SEQ ID NO: 119: synthetic nucleic acid
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Sequence Listing:

SEQUENCE LISTING

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aggtcttagg acaggccaga gccaagcttg agtcatggaa ggagggtccc tatacagtag 180

atgcaatcca aaagaaaatc acagaaacca ag 212

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ccggttctga aggtgttctt gta 23

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cctccggttc tgaaggtgtt cttgta 26

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ccggttctga aggtgttctt gt 22

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<400> SEQUENCE: 10

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<400> SEQUENCE: 21

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<210> SEQ ID NO 22
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<210> SEQ ID NO 23
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<212> TYPE: DNA
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cctccggttc tgaaggtgtt ctt 23

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<400> SEQUENCE: 24

gcctccggtt ctgaaggtgt tctt 24

<210> SEQ ID NO 25
<211> LENGTH: 25
<212> TYPE: DNA
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<400> SEQUENCE: 25

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<210> SEQ ID NO 26
<211> LENGTH: 19
<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 26

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<210> SEQ ID NO 27
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<220> FEATURE:
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<400> SEQUENCE: 27

tccggttctg aaggtgttct 20

<210> SEQ ID NO 28
<211> LENGTH: 21
<212> TYPE: DNA
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ctccggttct gaaggtgttc t 21

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cctccggttc tgaaggtgtt ct 22

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gcctccggtt ctgaaggtgt tct 23

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<400> SEQUENCE: 32

ccggttctga aggtgttc 18

<210> SEQ ID NO 33
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<400> SEQUENCE: 33

tccggttctg aaggtgttc 19

<210> SEQ ID NO 34
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<212> TYPE: DNA
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<400> SEQUENCE: 34

ctccggttct gaaggtgttc 20

<210> SEQ ID NO 35
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<400> SEQUENCE: 35

cctccggttc tgaaggtgtt c 21

<210> SEQ ID NO 36
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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 36

gcctccggtt ctgaaggtgt tc 22

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<212> TYPE: DNA
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<400> SEQUENCE: 37

tgcctccggt tctgaaggtg ttc 23

<210> SEQ ID NO 38
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 38

cattcaactg ttgcctccgg ttctgaaggt g 31

<210> SEQ ID NO 39
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 39

ttgcctccgg ttctgaaggt gttcttgtac 30

<210> SEQ ID NO 40
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<212> TYPE: DNA
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<400> SEQUENCE: 40

aggatttgga acagaggcgt c 21

<210> SEQ ID NO 41
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<212> TYPE: DNA
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gtctgccact ggcggaggtc 20

<210> SEQ ID NO 42
<211> LENGTH: 20
<212> TYPE: DNA
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<400> SEQUENCE: 42

catcaagcag aaggcaacaa 20

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<210> SEQ ID NO 43
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<212> TYPE: DNA
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<400> SEQUENCE: 43

gaagtttcag ggccaagtca 20

<210> SEQ ID NO 44
<211> LENGTH: 963
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 44

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tgctcctttg ccacaacgga cgacttctat gacgacccgt gtttcgactc cccggacctg 120

cgcttcttcg aagacctgga cccgcgcctg atgcacgtgg gcgcgctcct gaaacccgaa 180

gagcactcgc acttccccgc ggcggtgcac ccggccccgg gcgcacgtga ggacgagcat 240

gtgcgcgcgc ccagcgggca ccaccaggcg ggccgctgcc tactgtgggc ctgcaaggcg 300

tgcaagcgca agaccaccaa cgccgaccgc cgcaaggccg ccaccatgcg cgagcggcgc 360

cgcctgagca aagtaaatga ggcctttgag acactcaagc gctgcacgtc gagcaatcca 420

aaccagcggt tgcccaaggt ggagatcctg cgcaacgcca tccgctatat cgagggcctg 480

caggctctgc tgcgcgacca ggacgccgcg ccccctggcg ccgcagccgc cttctatgcg 540

ccgggcccgc tgcccccggg ccgcggcggc gagcactaca gcggcgactc cgacgcgtcc 600

agcccgcgct ccaactgctc cgacggcatg atggactaca gcggcccccc gagcggcgcc 660

cggcggcgga actgctacga aggcgcctac tacaacgagg cgcccagcga acccaggccc 720

gggaagagtg cggcggtgtc gagcctagac tgcctgtcca gcatcgtgga gcgcatctcc 780

accgagagcc ctgcggcgcc cgccctcctg ctggcggacg tgccttctga gtcgcctccg 840

cgcaggcaag aggctgccgc ccccagcgag ggagagagca gcggcgaccc cacccagtca 900

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tga 963

<210> SEQ ID NO 45
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<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 45

cgggcttgga cagaacttac 20

<210> SEQ ID NO 46
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 46

tccttacggg tagcatcctg 20

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<210> SEQ ID NO 47
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 47

ctgaaggtgt tcttgtactt catcc 25

<210> SEQ ID NO 48
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 48

tgttgagaaa tggcggcgt 19

<210> SEQ ID NO 49
<211> LENGTH: 31
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 49

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<210> SEQ ID NO 50
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<212> TYPE: RNA
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<220> FEATURE:
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<400> SEQUENCE: 50

ucccacugau ucugaauucu uucaa 25

<210> SEQ ID NO 51
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<400> SEQUENCE: 51

cuucauccca cugauucuga auucu 25

<210> SEQ ID NO 52
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<212> TYPE: RNA
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uuguacuuca ucccacugau ucuga 25

<210> SEQ ID NO 53
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<212> TYPE: RNA
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<400> SEQUENCE: 53

uguucuugua cuucauccca cugau 25

<210> SEQ ID NO 54
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<212> TYPE: RNA
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<400> SEQUENCE: 54

gaagguguuc uuguacuuca uccca 25

<210> SEQ ID NO 55
<211> LENGTH: 25
<212> TYPE: RNA
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guucugaagg uguucuugua cuuca 25

<210> SEQ ID NO 56
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<212> TYPE: RNA
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<220> FEATURE:
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<400> SEQUENCE: 56

cuccgguucu gaagguguuc uugua 25

<210> SEQ ID NO 57
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 57

guugccuccg guucugaagg uguuc 25

<210> SEQ ID NO 58
<211> LENGTH: 25
<212> TYPE: RNA
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<400> SEQUENCE: 58

caacuguugc cuccgguucu gaagg 25

<210> SEQ ID NO 59
<211> LENGTH: 25
<212> TYPE: RNA
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<220> FEATURE:
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<400> SEQUENCE: 59

ucauucaacu guugccuccg guucu 25

<210> SEQ ID NO 60
<211> LENGTH: 25

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<212> TYPE: RNA
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<220> FEATURE:
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<400> SEQUENCE: 60

acauuucauu caacuguugc cuccg 25

<210> SEQ ID NO 61
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<212> TYPE: RNA
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<400> SEQUENCE: 61

cuuuaacauu ucauucaacu guugc 25

<210> SEQ ID NO 62
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<212> TYPE: RNA
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<400> SEQUENCE: 62

gaauccuuua acauuucauu caacu 25

<210> SEQ ID NO 63
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<212> TYPE: RNA
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<220> FEATURE:
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<400> SEQUENCE: 63

guguugaauc cuuuaacauu ucauu 25

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<212> TYPE: RNA
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<220> FEATURE:
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<400> SEQUENCE: 64

ccauuguguu gaauccuuua acauu 25

<210> SEQ ID NO 65
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 65

uccagccauu guguugaauc cuuua 25

<210> SEQ ID NO 66
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 66

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uagcuuccag ccauuguguu gaauc 25

<210> SEQ ID NO 67
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<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 67

uuccuuagcu uccagccauu guguu 25

<210> SEQ ID NO 68
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<400> SEQUENCE: 68

gcuucuuccu uagcuuccag ccauu 25

<210> SEQ ID NO 69
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<212> TYPE: DNA
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<400> SEQUENCE: 69

gcucagcuuc uuccuuagcu uccag 25

<210> SEQ ID NO 70
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 70

gaccugcuca gcuucuuccu uagcu 25

<210> SEQ ID NO 71
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 71

ccuaagaccu gcucagcuuc uuccu 25

<210> SEQ ID NO 72
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 72

ccuguccuaa gaccugcuca gcuuc 25

<210> SEQ ID NO 73
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial

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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 73

ucuggccugu ccuaagaccu gcuca 25

<210> SEQ ID NO 74
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 74

uuggcucugg ccuguccuaa gaccu 25

<210> SEQ ID NO 75
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 75

caagcuuggc ucuggccugu ccuaa 25

<210> SEQ ID NO 76
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 76

ugacucaagc uuggcucugg ccugu 25

<210> SEQ ID NO 77
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uuccaugacu caagcuuggc ucugg 25

<210> SEQ ID NO 78
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<400> SEQUENCE: 78

ccuccuucca ugacucaagc uuggc 25

<210> SEQ ID NO 79
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<400> SEQUENCE: 79

gggacccucc uuccaugacu caagc 25

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<210> SEQ ID NO 80
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 80

guauagggac ccuccuucca ugacu 25

<210> SEQ ID NO 81
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 81

cuacuguaua gggacccucc uucca 25

<210> SEQ ID NO 82
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 82

ugcaucuacu guauagggac ccucc 25

<210> SEQ ID NO 83
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 83

uggauugcau cuacuguaua gggac 25

<210> SEQ ID NO 84
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 84

ucuuuuggau ugcaucuacu guaua 25

<210> SEQ ID NO 85
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<212> TYPE: RNA
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<220> FEATURE:
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cuccgguucu gaaggugu 18

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ugccuccggu ucugaagg 18

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uguugccucc gguucuga 18

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<400> SEQUENCE: 123

cuguugccuc cgguucug 18

The invention claimed is:

1. An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of the nucleotide sequence of SEQ ID NO: 35, wherein the antisense oligomer is an oligonucleotide having the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide modified, or a morpholino oligomer.

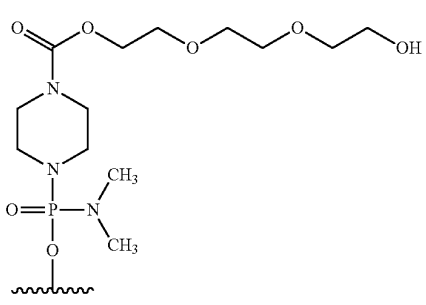
2. The antisense oligomer according to claim **1**, wherein the antisense oligomer is a morpholino oligomer.

3. The antisense oligomer according to claim **1**, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br and I (wherein R is an alkyl or an aryl and R' is an alkylene).

4. The antisense oligomer according to claim **1**, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond and a boranophosphate bond.

5. The antisense oligomer according to claim **2**, wherein the morpholino oligomer is a phosphorodiamidate morpholino oligomer.

6. The antisense oligomer according to claim **2**, wherein the 5 end of the morpholino oligomer is one of the groups of chemical formulae (1) to (3) below:



(1)

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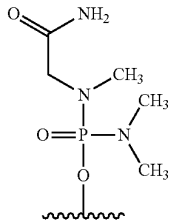
(2)



(3)

7. A pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active, ingredient the antisense oligomer according to claim **1**, or a pharmaceutically acceptable salt or hydrate thereof.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 9,079,934 B2
APPLICATION NO. : 13/819520
DATED : July 14, 2015
INVENTOR(S) : Naoki Watanabe et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Claims

At Column 84, Line 51, replace “the 5 end of” with --the 5’ end of--.

Signed and Sealed this
Twenty-third Day of June, 2020

Andrei Iancu
Director of the United States Patent and Trademark Office

EXHIBIT AB

Atty. Docket No.: 209658-0001-00-US-495293
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re U.S. Patent No. 9,079,934

Issued: July 14, 2015

To: Naoki WATANABE et al.

Assignees: NIPPON SHINYAKU CO., LTD. AND NATIONAL CENTER OF NEUROLOGY AND PSYCHIATRY

For: ANTISENSE NUCLEIC ACIDS

APPLICATION FOR EXTENSION OF PATENT TERM UNDER 35 U.S.C. § 156

MAIL STOP HATCH-WAXMAN PTE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Applicant, NIPPON SHINYAKU CO., LTD., represents that it is the Assignee of an undivided interest in the entirety in and to United States Patent No. 9,079,934 granted to Naoki Watanabe et al. on the July 14, 2015, for ANTISENSE NUCLEIC ACIDS by virtue of an assignment from the inventors to NIPPON SHINYAKU CO., LTD., recorded in the U.S. Patent and Trademark Office at Reel 030185, Frame 0302 on April 10, 2013.

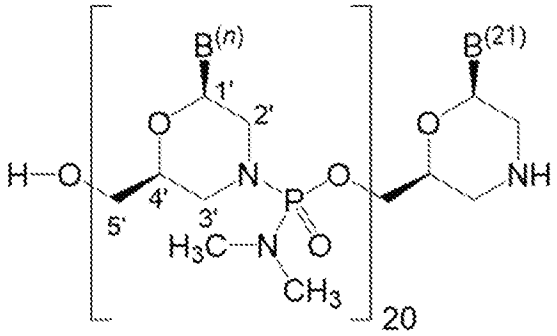
By the Power of Attorney submitted April 10, 2013, the inventors appoint the registered practitioners associated with Customer Number 055694, including Mercedes K. Meyer and Zhengyu Feng, as attorneys with regard to this application for extension of the patent term of U.S. Patent No. 9,079,934 and to transact all business in the U.S. Patent and Trademark Office in connection therewith.

Docket No.: 209658-0001-00-US-495293

Information Required Under 37 C.F.R. § 1.740

Applicant hereby submits this application for extension of the patent term under 35 U.S.C. § 156 by providing the following information required by the rules promulgated by the U.S. Patent and Trademark Office (37 C.F.R. § 1.740). For the convenience of the Patent and Trademark Office, the information contained in this application will be presented in a format that follows the requirements of Section 1.740 of Title 37 of the Code of Federal Regulations.

(1) The approved product, VILTEPSO™ (viltolarsen), is a small molecule. A chemical name of viltolarsen is: all-P-ambo-[2',3'-azanediyl-P,2',3'-trideoxy-P-(dimethylamino)-2',3'-seco](2'-N→5')(CCTCCGGTTC TGAAGGTGTT C). Viltolarsen is represented by the following structural formula:



CCTCCGGTTC TGAAGGTGTT C

(2) The approved product, VILTEPSO™ (viltolarsen), is subject to regulatory review under the Federal Food, Drug and Cosmetic Act Section 505, Part (b)(1).

(3) The approved product, VILTEPSO™ (viltolarsen), received approval for commercial marketing or use under Section 505, Part (b)(1) of the Federal Food, Drug and Cosmetic Act from the Food and Drug Administration on August 12, 2020.

(4) The active ingredient in VILTEPSO™ is viltolarsen, which on information and belief, has not been previously approved for commercial marketing or use under the Federal Food, Drug and Cosmetic Act, the Public Health Service Act, or the Virus-Serum-Toxin Act. A copy of the package insert describing the approved product is attached (Attachment A).

(5) This application for extension of patent term under 35 U.S.C. § 156 is being submitted within the permitted 60-day period pursuant to 37 C.F.R. § 1.720(f) (i.e., 60 days from August 12, 2020), said period will expire on October 11, 2020.

(6) The complete identification of the patent for which a term extension is being sought is as follows:

Inventors: Naoki Watanabe; Youhei Satou; Shin'ichi Takeda; and Tetsuya Nagata

Patent No.: 9,079,934

Application Serial No.: 13/819,520

Filing Date: August 31, 2011

Issue Date: July 14, 2015

Expiration Date: August 31, 2031

(7) A true copy of U.S. Patent 9,079,934 is attached (Attachment B).

(8) No disclaimer, reexamination certificate, or certificate of correction has been issued on this patent. A copy of the maintenance fee statement indicating payment of the fourth year maintenance fee on January 3, 2019 is attached (Attachment C).

(9) Claims 1-2 and 5-7 of U.S. Patent No. 9,079,934 read upon the approved product, VILTEPSO™ (viltolarsen).

Claim 1 reads as follows:

1. An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of the nucleotide sequence of SEQ ID NO: 35, wherein the antisense oligomer is an oligonucleotide having the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide modified, or a morpholino oligomer.

Viltolarsen is an antisense phosphorodiamidate morpholino oligomer. Viltolarsen is 21 nucleobases in length and has the following sequence: 5'- CCTCCGGTTC TGAAGGTGTT C -3', which corresponds to SEQ ID NO: 35 in U.S. Patent No. 9,079,934.

(10) The relevant dates and information pursuant to 35 U.S.C. § 156(g) to enable the Secretary of Health and Human Services to determine the applicable regulatory review period are as follows:

- Investigational New Drug Application (IND 127474) for VILTEPSO™ (viltolarsen) became effective on March 25, 2016.
- New Drug Application (NDA 212154) for VILTEPSO™ (viltolarsen) was filed on February 1, 2019.
- New Drug Application (NDA 212154) was approved on August 12, 2020.

(11) A brief description of the significant activities undertaken by the marketing applicant during the applicable regulatory review period with respect to the approved product and the dates applicable to these significant activities are set forth in a chronology of events in Attachment D.

(12)(i) Applicant is of the opinion that U.S. Patent No. 9,079,934 is eligible for extension of the patent term under 35 U.S.C. § 156.

(12)(ii) Applicant respectfully submits that the length of the extension of patent term for U.S. Patent No. 9,079,934 is **1,077** days pursuant to 35 U.S.C. § 156(c) (i.e., the difference between Aug. 31, 2031 and 14 years from the NDA approval date). The length of the extension was determined pursuant to 37 C.F.R. § 1.775(a) to (d) as follows:

1.775(c)(1)and (c)(2)

(a) The regulatory review period under 35 U.S.C. § 156(g)(1)(B) is a total of 1,603 days, which is the sum of (1) and (2) below:

(1) The period of review under 35 U.S.C. § 156(g)(1)(B)(i), the "Testing Period," began on March 25, 2016. The date the NDA was initially submitted is February 1, 2019. The difference between these dates is 1,044 days (1.775(c)(1)); and

(2) The period of review under 35 U.S.C. § 156(g)(1)(B)(ii), the "Approval Period," began on February 1, 2019, and ended on August 12, 2020, which is a total of 559 days (1.775(c)(2)).

1.775(d)(1)(i)-(iii)

(b) The regulatory review period upon which the period of extension is calculated is the entire regulatory review period as determined in subparagraph 12(ii)(a) above (1,603 days) less:

(1) The number of days in the regulatory review period which were before or on the date on which the patent issued (July 14, 2015), which is 0 days pursuant to 37 C.F.R. §1.775(d)(1)(i); there are no days known to applicant during the periods of 1.775(c)(1) and (2) above that applicant failed to act diligently, thus 0 days pursuant to 37 C.F.R. §1.775(d)(1)(ii);

(2) One-half the number of days determined in subparagraph (12)(ii)(a)(1) above after the patent issued which is (1044 days – 0 days)/2 or 522 days; the sum

results in a total regulatory review period upon which the period of extension is calculated of (1,603 days minus 0 days minus 522 days) or 1,081 days.

(c) The number of days as determined in subparagraph (12)(ii)(b) (i.e., 1,081 days) when added to the original term of the patent (August 31, 2031) would result in the expiration date of August 16, 2034. 37 C.F.R. 1.755(d)(2).

(d) Fourteen (14) years when added to the date of the NDA approval date (i.e., August 12, 2020 plus 14 years) would result in the expiration date of August 12, 2034. 37 C.F.R. 1.755(d)(3).

(e) The earlier date as determined in subparagraphs (12)(ii)(c) and (12)(ii)(d) is August 12, 2034. 37 C.F.R. 1.755(d)(4).

(f) Since U.S. Patent No. 9,079,934 issued after September 24, 1984, the period of extension may not exceed five years from the original expiration date of August 31, 2031 (The application was filed August 31, 2011 and there are 0 days added under 35 U.S.C. 154). Five years when added to the original expiration date of the patent would result in the date of August 31, 2036. 37 C.F.R. 1.755(d)(5).

(g) The earlier date as determined by subparagraphs (12)(ii)(e) and (12)(ii)(f) is August 12, 2034.

(13) Applicant acknowledges a duty to disclose to the Director of the United States Patent and Trademark Office and the Secretary of Health and Human Services any information which is material to the determination of entitlement to the extension sought pursuant to §§1.704(a)(13) and 1.765.

(14) Please charge the prescribed fee for receiving and acting upon this application in the amount of $**1,180.00** to Deposit Account No. 50-0573. The Director is authorized to charge any additional fees required by this application to Deposit Account No. 50-0573.

(15) (A) All correspondence and inquiries may be directed to the undersigned at the correspondence address associated with Customer No. 055694.

(15) (B) This is a certification that the application for extension of patent term under 35 U.S.C. § 156 including its attachments and supporting papers is being submitted via EFS-Web given the USPTO's official notice of May 29, 2020 (https://www.uspto.gov/about-us/news-

updates/uspto-allow-filing-initial-patent-term-extension-applications-patent). The notice of May 29th waived the requirement of submitting one original application and two copies for the total of three copies under 1.740(a)(12) given the 2020 coronavirus pandemic. The submitted EFS application can also be considered as having been submitted in triplicate for purposes of intent.

Applicant submits based on the above calculations that Applicant is entitled to 1,077 days of patent term extension.

Dated: October 9, 2020

Respectfully submitted,

Customer Number: 055694

By: /Mercedes K. Meyer/

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Registration No.: 44,939
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Attorneys/Agents For Applicant

Attachments:
Approved Package Insert (Attachment A)
U.S. Patent No. 9,079,934 (Attachment B)
Maintenance Fee Statement (Attachment C)
Chronology of Regulatory Review Period (Attachment D)

ATTACHMENT A

HIGHLIGHTS OF PRESCRIBING INFORMATION

These highlights do not include all the information needed to use VILTEPSO™ safely and effectively. See full prescribing information for VILTEPSO.

VILTEPSO (viltolarsen) injection, for intravenous use
Initial U.S. Approval: 2020

----------INDICATIONS AND USAGE----------

VILTEPSO is an antisense oligonucleotide indicated for the treatment of Duchenne muscular dystrophy (DMD) in patients who have a confirmed mutation of the DMD gene that is amenable to exon 53 skipping. This indication is approved under accelerated approval based on an increase in dystrophin production in skeletal muscle observed in patients treated with VILTEPSO. Continued approval for this indication may be contingent upon verification and description of clinical benefit in a confirmatory trial. (1)

----------DOSAGE AND ADMINISTRATION----------

- Serum cystatin C, urine dipstick, and urine protein-to-creatinine ratio should be measured before starting VILTEPSO. (2.1)
- Recommended dosage is 80 milligrams per kilogram of body weight once weekly. (2.2)
- Administer as an intravenous infusion over 60 minutes. (2.2, 2.4)
- If the volume of VILTEPSO required is less than 100 mL, dilution in 0.9% Sodium Chloride Injection, USP, is required. (2.3)

----------DOSAGE FORMS AND STRENGTHS----------

Injection: 250 mg/5 mL (50 mg/mL) in a single-dose vial (3)

----------CONTRAINDICATIONS----------

None (4)

----------WARNINGS AND PRECAUTIONS----------

Kidney Toxicity: Based on animal data, may cause kidney toxicity. Kidney function should be monitored; creatinine may not be a reliable measure of renal function in DMD patients. (5.1, 13.2)

----------ADVERSE REACTIONS----------

The most common adverse reactions (incidence ≥15% in patients treated with VILTEPSO) were upper respiratory tract infection, injection site reaction, cough, and pyrexia. (6.1)

To report SUSPECTED ADVERSE REACTIONS, contact NS Pharma at 1-866 NSPHARM (1-866-677-4276) or FDA at 1-800-FDA-1088 or *www.fda.gov/medwatch*.

See 17 for PATIENT COUNSELING INFORMATION

Revised: 8/2020

FULL PRESCRIBING INFORMATION: CONTENTS*

* Sections or subsections omitted from the full prescribing information are not listed.

FULL PRESCRIBING INFORMATION

1 INDICATIONS AND USAGE

VILTEPSO is indicated for the treatment of Duchenne muscular dystrophy (DMD) in patients who have a confirmed mutation of the DMD gene that is amenable to exon 53 skipping. This indication is approved under accelerated approval based on an increase in dystrophin production in skeletal muscle observed in patients treated with VILTEPSO *[see Clinical Studies (14)].* Continued approval for this indication may be contingent upon verification and description of clinical benefit in a confirmatory trial.

2 DOSAGE AND ADMINISTRATION

2.1 Monitoring to Assess Safety

Serum cystatin C, urine dipstick, and urine protein-to-creatinine ratio should be measured before starting VILTEPSO. Consider measurement of glomerular filtration rate prior to initiation of VILTEPSO. Monitoring for kidney toxicity during treatment is recommended *[see Warnings and Precautions (5.1)].*

2.2 Dosing Information

The recommended dosage of VILTEPSO is 80 mg/kg administered once weekly as a 60-minute intravenous infusion.

If a dose of VILTEPSO is missed, it should be administered as soon as possible after the scheduled dose time.

2.3 Preparation Instructions

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit. Prepare the VILTEPSO dose using aseptic technique.

a. Calculate the total dose of VILTEPSO to be administered based on the patient's weight and the recommended dosage of 80 mg/kg. Determine the volume of VILTEPSO needed and the correct number of vials to supply the full calculated dose.
b. Allow vials to warm to room temperature. Mix the contents of each vial by gently inverting 2 to 3 times. Do not shake.
c. Visually inspect each vial of VILTEPSO. VILTEPSO is a clear and colorless solution. Do not use if the solution in the vials is discolored or particulate matter is present.
d. Withdraw the calculated volume of VILTEPSO from the appropriate number of vials.
    i. If the volume of VILTEPSO required is less than 100 mL, dilution in 0.9% Sodium Chloride Injection, USP is required. Withdraw from the 100-mL infusion bag a volume of 0.9% Sodium Chloride Injection, USP, equivalent to the calculated volume of VILTEPSO and inject the VILTEPSO into the infusion bag, such that the total volume in the bag is 100 mL.
    ii. If the volume of VILTEPSO required is 100 mL or more, dilution is not required, and the required amount of VILTEPSO should be placed into an empty infusion bag.

e. Visually inspect the infusion bag containing the solution for particulates. Gently invert the infusion bag to ensure equal distribution of product. Do not shake.
f. VILTEPSO contains no preservatives. Infusion should begin as soon as possible, but no more than 5 hours after preparation of VILTEPSO, and be completed within 6 hours of preparation (allowing for 1 hour of infusion time), if diluted solution is stored at 20°C to 26°C (68°F to 79°F). If immediate use is not possible, the solution may be stored for up to 24 hours at 2°C to 8°C (36°F to 46°F). Do not freeze.
g. VILTEPSO is supplied in single-dose vials. Discard unused VILTEPSO.

2.4 Administration Instructions

VILTEPSO is administered via intravenous infusion using a peripheral or central venous catheter. Flush the intravenous access line with 0.9% Sodium Chloride Injection, USP, after infusion. Filtration of VILTEPSO is not required.

Infuse VILTEPSO over 60 minutes. Do not mix other medications with VILTEPSO or infuse other medications concomitantly via the same intravenous access line. VILTEPSO should be mixed with 0.9% Sodium Chloride Injection, USP, only.

3 DOSAGE FORMS AND STRENGTHS

VILTEPSO is a clear and colorless solution available as follows:

- Injection: 250 mg/5 mL (50 mg/mL) solution in a single-dose vial

4 CONTRAINDICATIONS

None.

5 WARNINGS AND PRECAUTIONS

5.1 Kidney Toxicity

Kidney toxicity was observed in animals who received viltolarsen *[see Use in Specific Populations (8.4)].* Although kidney toxicity was not observed in the clinical studies with VILTEPSO, the clinical experience with VILTEPSO is limited, and kidney toxicity, including potentially fatal glomerulonephritis, has been observed after administration of some antisense oligonucleotides. Kidney function should be monitored in patients taking VILTEPSO. Because of the effect of reduced skeletal muscle mass on creatinine measurements, serum creatinine may not be a reliable measure of kidney function in DMD patients. Serum cystatin C, urine dipstick, and urine protein-to-creatinine ratio should be measured before starting VILTEPSO. Consider also measuring glomerular filtration rate using an exogenous filtration marker before starting VILTEPSO. During treatment, monitor urine dipstick every month, and serum cystatin C and urine protein-to-creatinine ratio every three months. If a persistent increase in serum cystatin C or proteinuria is detected, refer to a pediatric nephrologist for further evaluation.

6 ADVERSE REACTIONS

6.1 Clinical Trials Experience

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

In clinical trials with VILTEPSO, 32 patients have been exposed to VILTEPSO once weekly, ranging between 40 mg/kg (0.5 times the recommended dosage) and 80 mg/kg (the recommended dosage), including 16 patients treated for greater than 12 months and 8 patients treated for greater than 24 months as part of an ongoing open-label extension study. All patients were male and had genetically confirmed DMD.

Study 1 was a multicenter, 2-period, dose-finding study conducted in the United States and Canada in males 4 years to less than 10 years of age on a stable corticosteroid regimen for at least 3 months. During the initial period (first 4 weeks) of Study 1, patients were randomized (double-blind) to VILTEPSO or placebo. All patients then received 20 weeks of VILTEPSO 40 mg/kg once weekly (0.5 times the recommended dose) (N=8), or 80 mg/kg once weekly (N=8) *[see Clinical Studies (14)].*

Study 2 was a multicenter, parallel-group, open-label, dose-finding study conducted in Japan. Eligible patients included ambulatory and non-ambulatory males 5 years to less than 18 years of age who were assigned to receive intravenous VILTEPSO 40 mg/kg once weekly (0.5 times the recommended dose) (N=8) or 80 mg/kg once weekly (N=8) for 24 weeks.

Adverse reactions reported in ≥10% of patients treated with VILTEPSO 80 mg/kg/wk in pooled Studies 1 and 2 are displayed in Table 1. The most common adverse reactions (incidence ≥15% in patients treated with VILTEPSO) were upper respiratory tract infection, injection site reaction, cough, and pyrexia. Patients in the pooled analysis were treated with VILTEPSO for 20 to 24 weeks.

Table 1: Adverse Reactions Reported in ≥10% of DMD Patients Treated with VILTEPSO 80 mg/kg Once Weekly (Pooled Studies 1 and 2)

Adverse Reaction	VILTEPSO 80 mg/kg Once Weekly (n=16) %
Upper respiratory tract infection*	63
Injection site reaction**	25
Cough	19
Pyrexia	19
Contusion	13
Arthralgia	13
Diarrhea	13
Vomiting	13
Abdominal pain	13
Ejection fraction decreased	13
Urticaria	13

* Upper respiratory tract infection includes the following terms: upper respiratory tract infection, nasopharyngitis, and rhinorrhea.
** Injection site reaction includes the following terms: injection site bruising, injection site erythema, injection site reaction, and injection site swelling.

6.2 Immunogenicity

As with all oligonucleotides, there is potential for immunogenicity. The detection of antibody formation is highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of antibody positivity in an assay may be influenced by several factors, including assay methodology, sample handling, timing of sample collection, concomitant medications, and underlying disease. For these reasons, comparison of the incidence of antibodies in the studies described below with the incidence of antibodies in other studies may be misleading.

For Study 1, samples collected from all 16 patients at Day 1 (pre-dose), Week 5, Week 13, and Week 24 were assessed for anti-viltolarsen antibodies. All samples were determined to be antibody negative. For the same study, serum samples collected from all 16 patients at Day 1 (pre-dose), Week 13, and Week 24 were analyzed for anti-dystrophin antibodies. Anti-dystrophin antibodies were detected in 1 out of 16 patients (6.25%) at Weeks 13 and 24; however, at Weeks 37, 49, 73, and 97, no anti-dystrophin antibodies were detected in the same patient. Further, this patient achieved a change from baseline in dystrophin levels that was comparable to the mean change in his dosage group (80 mg/kg/week) and there were no adverse events reported with this antibody production. For Study 2, all samples collected from the 16 patients were determined to be both anti-viltolarsen antibody and anti-dystrophin antibody negative. Overall, there was a lack of observed immunogenicity, which indicates that viltolarsen is not highly immunogenic.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Risk Summary

There are no human or animal data available to assess the use of VILTEPSO during pregnancy. In the U.S. general population, major birth defects occur in 2 to 4%, and miscarriage occurs in 15 to 20% of clinically recognized pregnancies.

8.2 Lactation

Risk Summary

There are no human or animal data to assess the effect of VILTEPSO on milk production, the presence of viltolarsen in milk, or the effects of VILTEPSO on the breastfed infant.

The developmental and health benefits of breastfeeding should be considered along with the mother's clinical need for VILTEPSO and any potential adverse effects on the breastfed infant from VILTEPSO or from the underlying maternal condition.

8.4 Pediatric Use

VILTEPSO is indicated for the treatment of DMD in patients who have a confirmed mutation of the DMD gene that is amenable to exon 53 skipping, including pediatric patients *[see Clinical Studies (14)]*.

Juvenile Animal Toxicity Data

Viltolarsen (0, 15, 60, 240, or 1200 mg/kg) was administered to juvenile male mice by subcutaneous injection on postnatal day (PND) 7 and by intravenous injection weekly from PND 14 to PND 70. The highest dose resulted in deaths because of renal toxicity. In surviving animals at 240 and 1200 mg/kg, there was a dose-dependent increase in the incidence and severity of renal tubular effects (including degeneration), which were not accompanied by clinical pathology correlates. Reduced body weight gain and delayed sexual maturation were observed at the highest dose tested. At the no-effect dose for renal toxicity (60 mg/kg), plasma exposures were similar to that in humans at the recommended human dose of 80 mg/kg/week.

8.5 Geriatric Use

DMD is largely a disease of children and young adults; therefore, there is no geriatric experience with VILTEPSO.

8.6 Patients with Renal Impairment

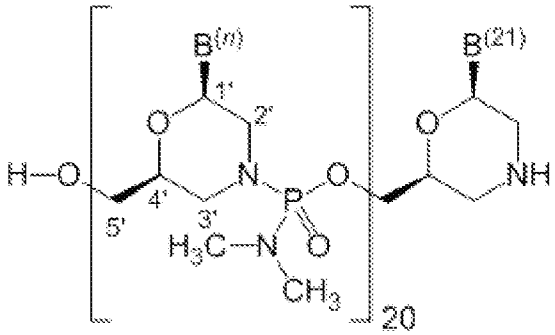
VILTEPSO has not been studied in patients with renal impairment. Viltolarsen is mostly excreted unchanged in the urine, and renal impairment may increase its exposure. However, because of the effect of reduced skeletal muscle mass on creatinine measurements in DMD patients, no specific dosage adjustment can be recommended for DMD patients with renal impairment based on estimated glomerular filtration rate. Patients with known renal function impairment should be closely monitored during treatment with VILTEPSO.

11 DESCRIPTION

VILTEPSO (viltolarsen) injection is a sterile, preservative-free, aqueous solution for intravenous administration. VILTEPSO is a clear and colorless solution. VILTEPSO is supplied in single-dose vials containing 250 mg/5 mL viltolarsen (50 mg/mL) in 0.9% sodium chloride. Each milliliter of VILTEPSO contains 50 mg viltolarsen and 9 mg sodium chloride in water for injection. The final product is adjusted to a pH ranging between 7.0 and 7.5 using hydrochloric acid and/or sodium hydroxide.

Viltolarsen is an antisense oligonucleotide of the phosphorodiamidate morpholino oligomer (PMO) subclass. PMOs are synthetic molecules in which the five-membered ribofuranosyl rings found in natural DNA and RNA are replaced by a six-membered morpholino ring. Each morpholino ring is linked through an uncharged phosphorodiamidate moiety rather than the negatively charged phosphate linkage that is present in natural DNA and RNA. Each phosphorodiamidate morpholino subunit contains one of the heterocyclic bases found in DNA (adenine, cytosine, guanine, or thymine). Viltolarsen contains 21 linked subunits. The molecular formula of viltolarsen is $C_{244}H_{381}N_{113}O_{88}P_{20}$ and the molecular weight is 6924.82 daltons. The structure and base sequence of viltolarsen are shown in Figure 1.

Figure 1: Structural Formula of Viltolarsen



CCTCCGGTTC TGAAGGTGTT C

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

VILTEPSO is designed to bind to exon 53 of dystrophin pre-mRNA resulting in exclusion of this exon during mRNA processing in patients with genetic mutations that are amenable to exon 53 skipping. Exon 53 skipping is intended to allow for production of an internally truncated dystrophin protein in patients with genetic mutations that are amenable to exon 53 skipping.

12.2 Pharmacodynamics

After treatment with VILTEPSO 80 mg/kg once weekly, all patients evaluated (N=8) were found to produce mRNA for a truncated dystrophin protein, as measured by reverse transcription polymerase chain reaction (RT-PCR), and demonstrated exon 53 skipping, as measured by DNA sequence analysis.

In Study 1, all patients who received VILTEPSO 80 mg/kg once weekly for 20 to 24 weeks showed an increase from baseline in dystrophin protein expression, as quantified by a validated Western blot method (mean 5.3%; median 3.8%; range 0.7% to 13.9% of normal levels when normalized to myosin heavy chain; p-value 0.01). Mass spectrometry, immunofluorescence staining, and RT-PCR results were supportive of the Western blot data *[see Clinical Studies (14)].* Expected localization of truncated dystrophin to the sarcolemma in muscle fibers of patients treated with viltolarsen was confirmed by immunofluorescence staining.

12.3 Pharmacokinetics

The pharmacokinetics of viltolarsen was evaluated in DMD patients following administration of intravenous (IV) doses ranging from 1.25 mg/kg/week (0.016 times the recommended dosage) to 80 mg/kg/week (the recommended dosage). Viltolarsen exposure increased proportionally with dose, with minimal accumulation with once-weekly dosing. Inter-subject variability (as %CV) for C_{max} and AUC ranged from 16% to 27% respectively.

VILTEPSO is administered as an IV infusion over 60 minutes. Bioavailability is assumed to be 100%, and median T_{max} was around 1 hour (end of infusion).

Distribution

The mean viltolarsen steady-state volume of distribution was 300 mL/kg (%CV=14 at a dose of 80 mg/kg. Viltolarsen plasma protein binding ranged from 39% to 40% and is not concentration dependent.

Elimination

Metabolism

Data from in vitro metabolism indicate that viltolarsen is metabolically stable. No metabolites were detected in plasma or urine.

Excretion

VILTEPSO is excreted mainly as an unchanged drug in the urine. Viltolarsen elimination half-life was 2.5 (%CV=8) hours, and plasma clearance was 217 mL/hr/kg (%CV=22).

Specific Populations

Age, Sex & Race

The pharmacokinetics of viltolarsen have been evaluated only in male pediatric DMD patients.There is no experience with VILTEPSO in patients 65 years of age or older. No marked differences in any PK parameters were observed between White and Asian patients.

Patients with Renal or Hepatic Impairment

VILTEPSO has not been studied in patients with renal or hepatic impairment. Viltolarsen was found to be metabolically stable, and hepatic metabolism does not contribute to the elimination of viltolarsen. In addition, viltolarsen was mainly excreted unchanged in the urine. Viltolarsen is eliminated renally, and renal impairment is expected to result in increasing exposure of viltolarsen. However, because of the effect of reduced skeletal muscle mass on creatinine measurements in DMD patients, no specific dosage adjustment can be recommended for DMD patients with renal impairment based on glomerular filtration rate estimated by serum creatinine *[see Use in Specific Populations (8.6)].*

In Vitro Drug Interaction Studies

Viltolarsen did not inhibit CYP3A4/5, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, UGT1A1, or UGT2B7. Viltolarsen did not induce CYP1A2, CYP2B6, or CYP3A4.

Viltolarsen is not metabolized by CYP enzymes and is not a substrate of transporters BCRP, BSEP, MDR1, OAT1, OAT3, OCT1, OCT2, MATE1, or MATE2-K. Viltolarsen did not inhibit the transporters tested (OATP1B1, OATP1B3, OAT3, BCRP, MDR1, BSEP, OAT1, OCT1, OCT2, MATE1, and MATE2-K).

Based on in vitro data, viltolarsen has a low potential for drug-drug interactions with major CYP enzymes and drug transporters in humans.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

Carcinogenesis

Carcinogenicity studies of viltolarsen have not been conducted.

Mutagenesis

Viltolarsen was negative for genotoxicity in *in vitro* (bacterial reverse mutation, chromosomal aberration in Chinese hamster lung cells) and *in vivo* (mouse bone marrow micronucleus) assays.

Impairment of Fertility

Intravenous administration of viltolarsen (0, 60, 240, or 1000 mg/kg) to male mice weekly prior to and during mating to untreated females did not have adverse effects on fertility. Plasma exposure (AUC) at the highest dose was approximately 18 times that in humans at the recommended human dose of 80 mg/kg/week.

14 CLINICAL STUDIES

The effect of VILTEPSO on dystrophin production was evaluated in one study in DMD patients with a confirmed mutation of the DMD gene that is amenable to exon 53 skipping (Study 1; NCT02740972).

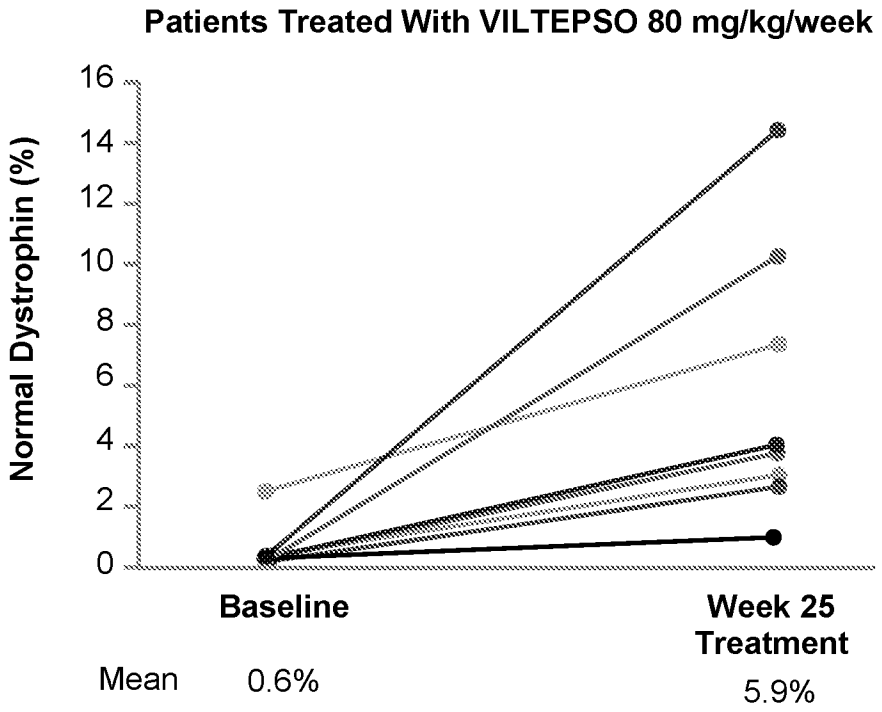
Study 1 was a multicenter, 2-period, dose-finding study conducted in the United States and Canada. During the initial period (first 4 weeks) of Study 1, patients were randomized (double blind) to VILTEPSO or placebo. All patients then received 20 weeks of open-label VILTEPSO 40 mg/kg once weekly (0.5 times the recommended dosage) (N=8) or 80 mg/kg once weekly (N=8). Study 1 enrolled ambulatory male patients 4 years to less than 10 years of age (median age 7 years) on a stable corticosteroid regimen for at least 3 months.

Efficacy was assessed based on change from baseline in dystrophin protein level (measured as % of the dystrophin level in healthy subjects, i.e., % of normal) at Week 25. Muscle biopsies (left or right biceps brachii) were collected from patients at baseline and following 24 weeks of VILTEPSO treatment, and analyzed for dystrophin protein level by Western blot normalized to myosin heavy chain (primary endpoint) and mass spectrometry (secondary endpoint).

In patients who received VILTEPSO 80 mg/kg once weekly, mean dystrophin levels increased from 0.6% (SD 0.8) of normal at baseline to 5.9% (SD 4.5) of normal by Week 25, with a mean change in dystrophin of 5.3% (SD 4.5) of normal levels (p=0.01) as assessed by validated Western blot (normalized to myosin heavy chain); the median change from baseline was 3.8%. All patients demonstrated an increase in dystrophin levels over their baseline values. As assessed by mass spectrometry (normalized to filamin C), mean dystrophin levels increased from 0.6% (SD 0.2) of normal at baseline to 4.2% (SD 3.7) of normal by Week 25, with a mean change in dystrophin of 3.7% (SD 3.8) of normal levels (nominal p=0.03, not adjusted for multiple comparisons); the median change from baseline was 1.9%.

Individual patient dystrophin levels in patients evaluated in Study 1 are shown in Figure 2 and Table 2.

Figure 2: Dystrophin Expression in Individual Patients (Study 1)



Note: Solid lines represent individual patient data. Dystrophin was measured using Western blot and normalized to myosin heavy chain.

Table 2: Dystrophin Expression in Individual Patients (Study 1)

Patient Number	Western Blot % Normal Dystrophin[a]		
	Baseline	Week 25	Change from Baseline
1	0.46	1.14	0.69
2	0.40	3.97	3.57
3	0.46	2.97	2.51
4	0.09	10.40	10.31
5	0.51	14.42	13.91
6	2.61	7.40	4.79
7	0.43	3.06	2.63
8	0.09	4.07	3.98

[a] Data were normalized by myosin heavy chain

16 HOW SUPPLIED/STORAGE AND HANDLING

16.1 How Supplied

VILTEPSO injection is supplied in single-dose vials. The solution is clear and colorless.

- Single-dose vials containing 250 mg/5 mL (50 mg/mL) viltolarsen NDC 73292-011-01

16.2 Storage and Handling

Store VILTEPSO at 2°C to 8°C (36°F to 46°F). Do not freeze.

17 Patient Counseling Information

Kidney Toxicity

Inform patients nephrotoxicity has occurred with drugs similar to VILTEPSO. Advise patients of the importance of monitoring for kidney toxicity by their healthcare providers during treatment with VILTEPSO *[see Warnings and Precautions (5.1)].*

Manufactured for:
NS Pharma, Inc.
Paramus, NJ 07652

This is a representation of an electronic record that was signed electronically. Following this are manifestations of any and all electronic signatures for this electronic record.

/s/

WILLIAM H Dunn
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ATTACHMENT B

US009079934B2

(12) **United States Patent**
Watanabe et al.

(10) **Patent No.: US 9,079,934 B2**
(45) **Date of Patent: Jul. 14, 2015**

(54) **ANTISENSE NUCLEIC ACIDS**

(75) Inventors: **Naoki Watanabe**, Tsukuba (JP); **Youhei Satou**, Tsukuba (JP); **Shin'ichi Takeda**, Tokyo (JP); **Tetsuya Nagata**, Tokyo (JP)

(73) Assignees: **NIPPON SHINYAKU CO., LTD.**, Kyoto-shi, Kyoto (JP); **NATIONAL CENTER OF NEUROLOGY AND PSYCHIATRY**, Kodaira-shi, Tokyo (JP)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **13/819,520**

(22) PCT Filed: **Aug. 31, 2011**

(86) PCT No.: **PCT/JP2011/070318**

§ 371 (c)(1),
(2), (4) Date: **Apr. 10, 2013**

(87) PCT Pub. No.: **WO2012/029986**

PCT Pub. Date: **Mar. 8, 2012**

(65) **Prior Publication Data**

US 2013/0211062 A1 Aug. 15, 2013

(30) **Foreign Application Priority Data**

Sep. 1, 2010 (JP) 2010-196032

(51) **Int. Cl.**

C07H 21/02	(2006.01)
C07H 21/04	(2006.01)
A61K 31/70	(2006.01)
C12N 15/11	(2006.01)
C12N 15/113	(2010.01)
C07H 21/00	(2006.01)
C12Q 1/68	(2006.01)

(52) **U.S. Cl.**

CPC ***C07H 21/04*** (2013.01); ***C07H 21/00*** (2013.01); ***C12N 15/111*** (2013.01); ***C12N 15/113*** (2013.01); *C12N 2310/11* (2013.01); *C12N 2310/315* (2013.01); *C12N 2310/321* (2013.01); *C12N 2310/3525* (2013.01); *C12N 2320/33* (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

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Primary Examiner — Sean McGarry

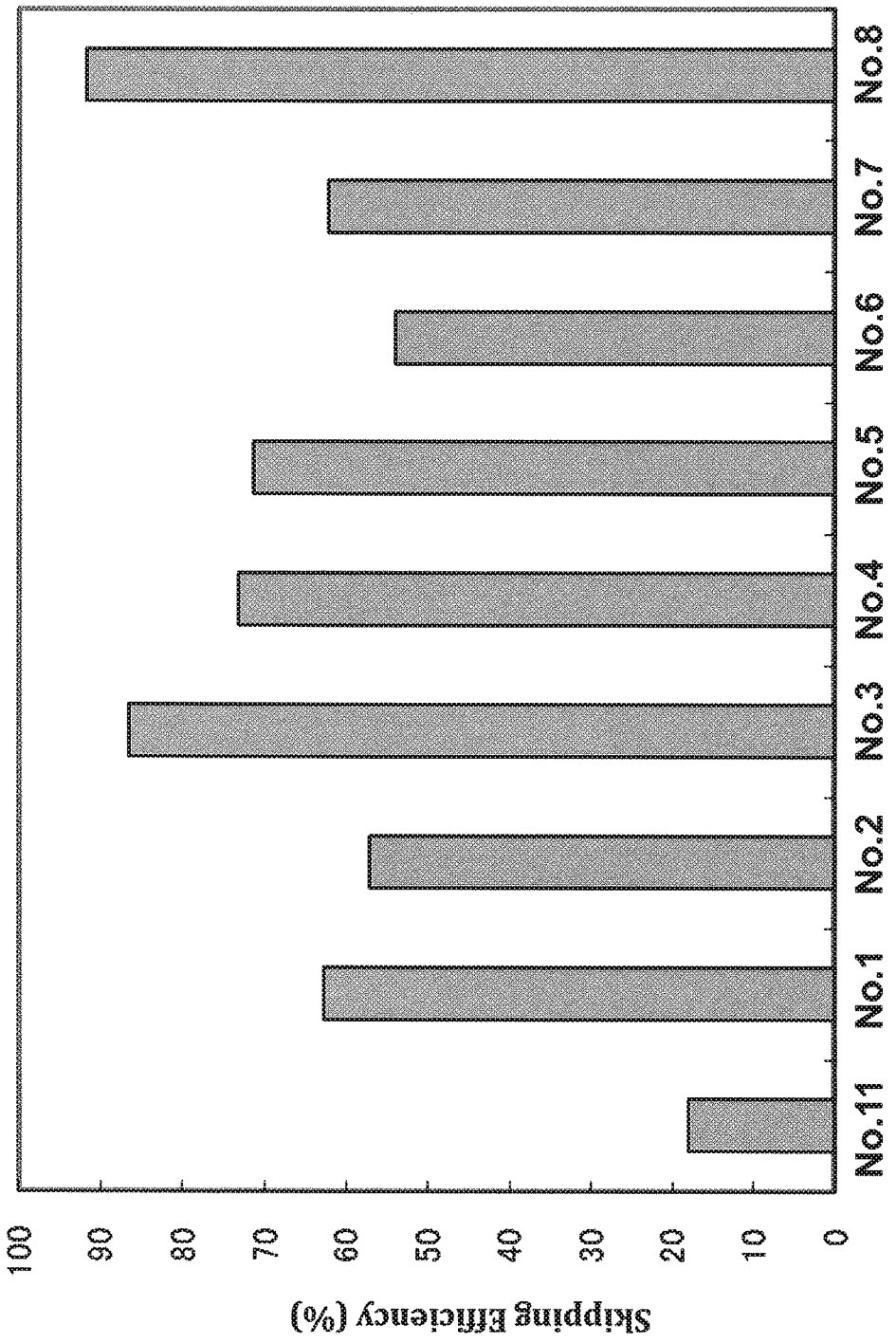
(74) *Attorney, Agent, or Firm* — Drinker Biddle & Reath LLP

(57) **ABSTRACT**

The present invention provides an oligomer which efficiently enables to cause skipping of the 53rd exon in the human dystrophin gene. Also provided is a pharmaceutical composition which causes skipping of the 53rd exon in the human dystrophin gene with a high efficiency.

7 Claims, 19 Drawing Sheets

Figure 1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Skipping Efficiency (%)

Figure 2

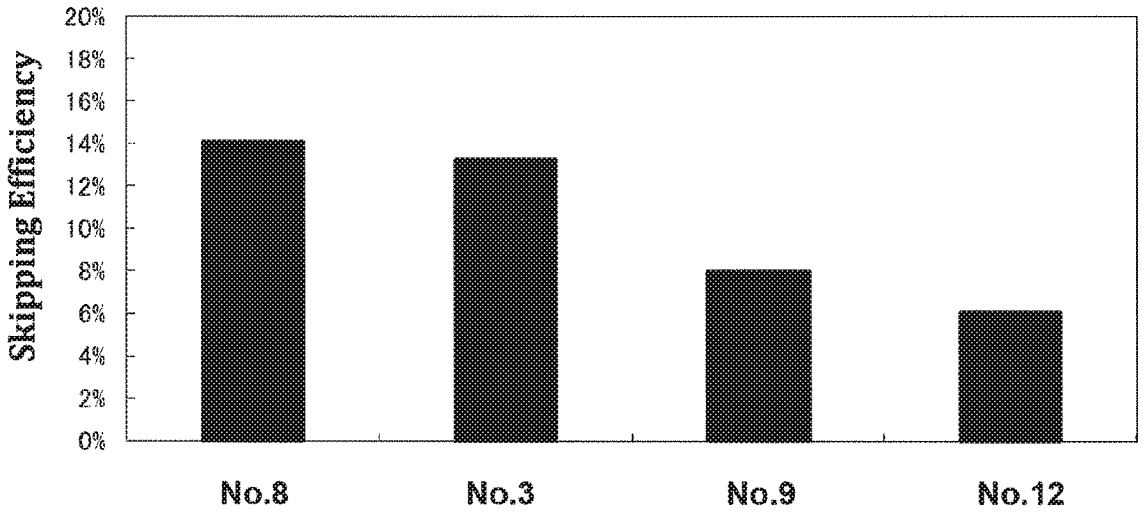
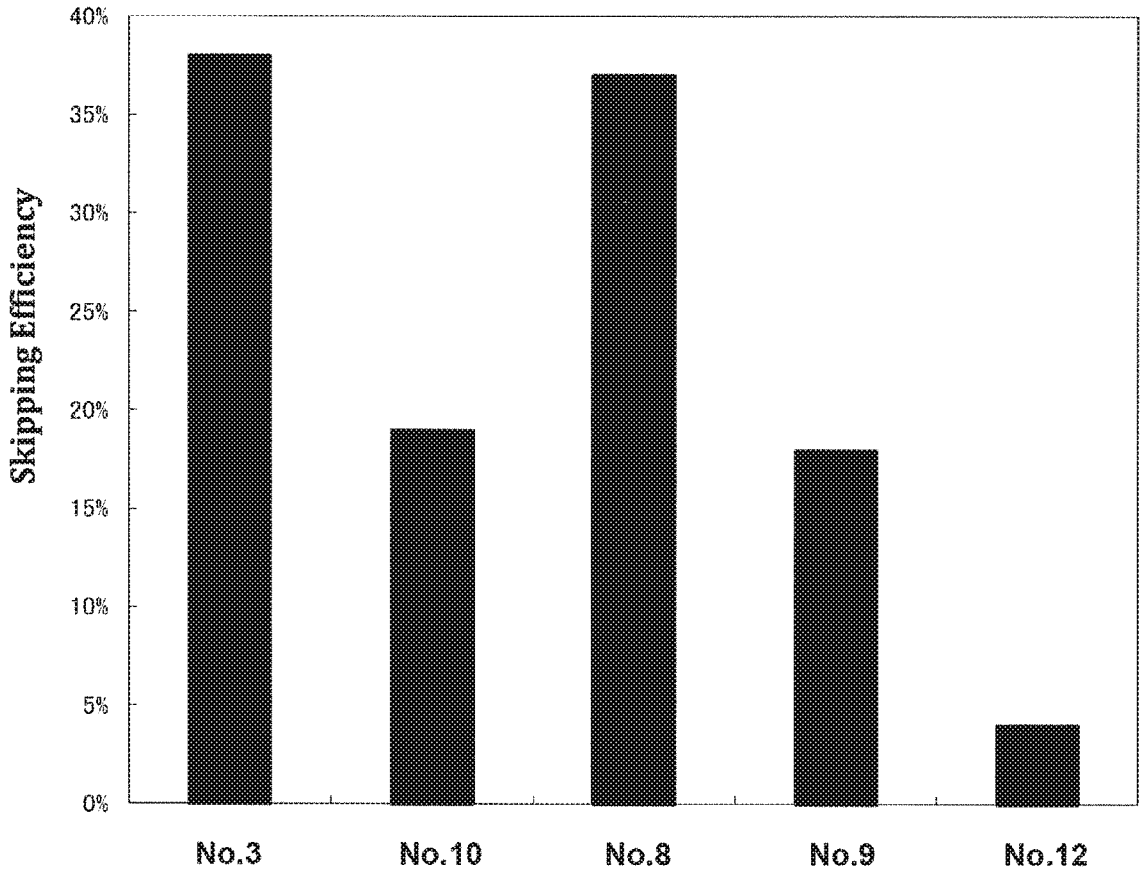


Figure 3


Skipping Efficiency
40%
35%
30%
25%
20%
15%
10%
5%
0%
No.3
No.10
No.8
No.9
No.12

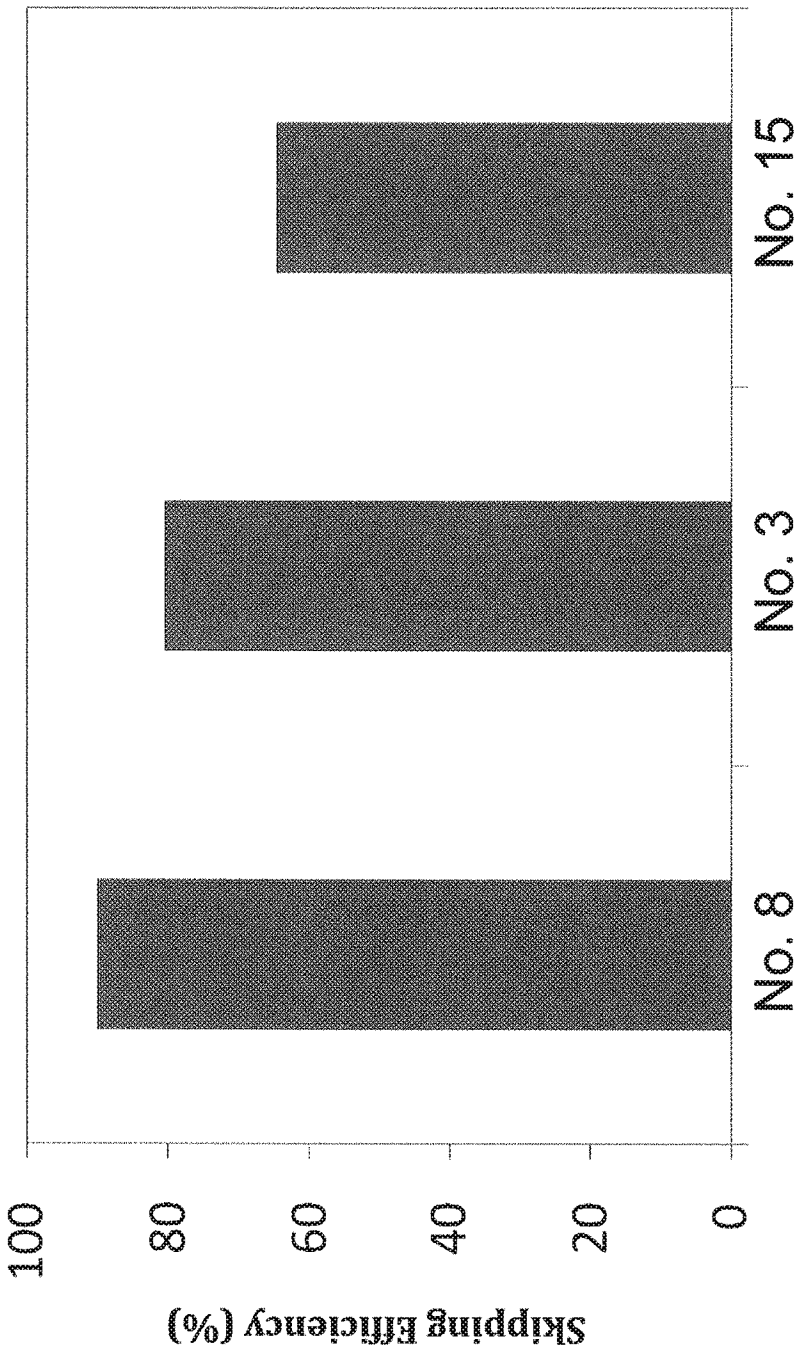


Figure 4

Figure 5

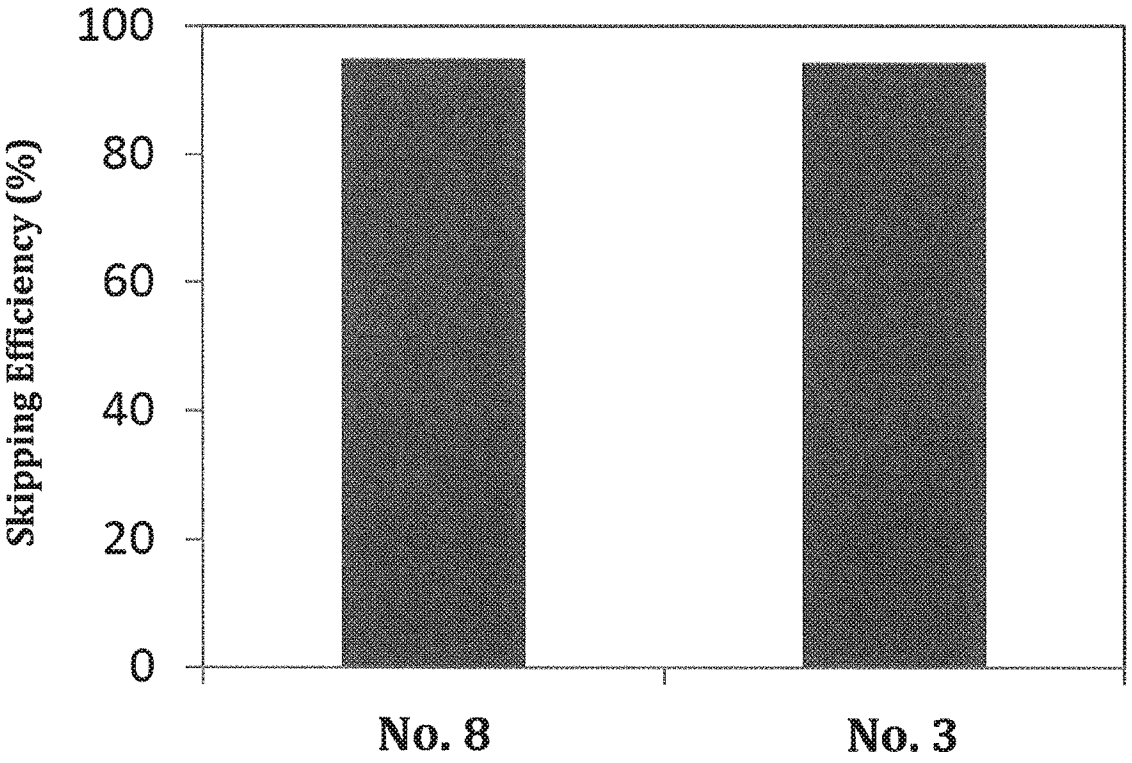


Figure 6

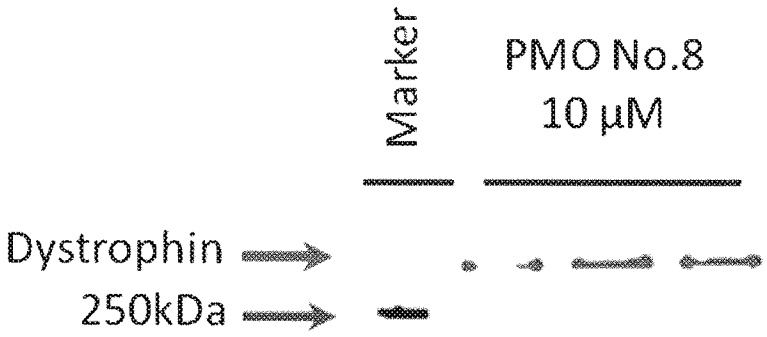


Figure 7

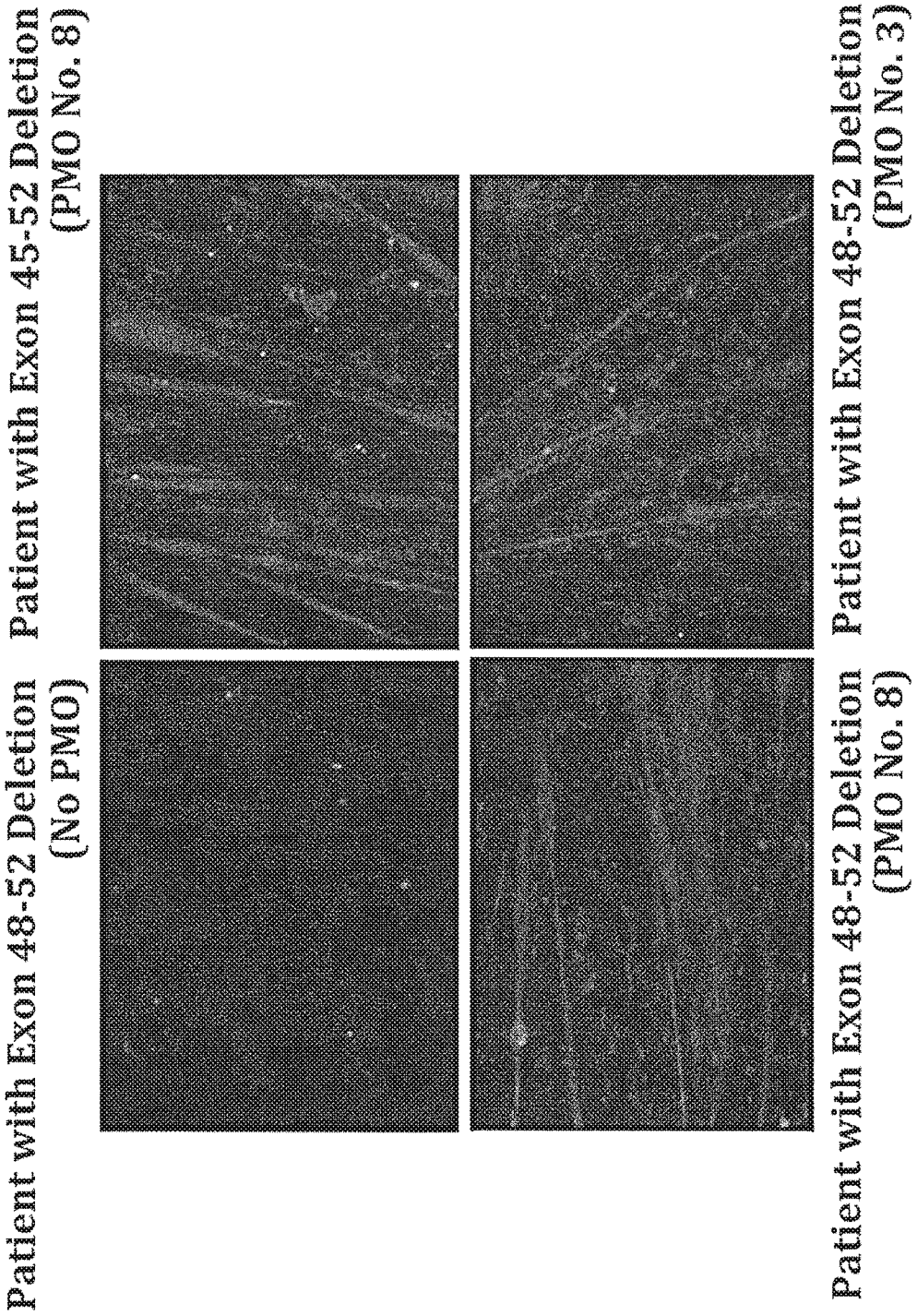
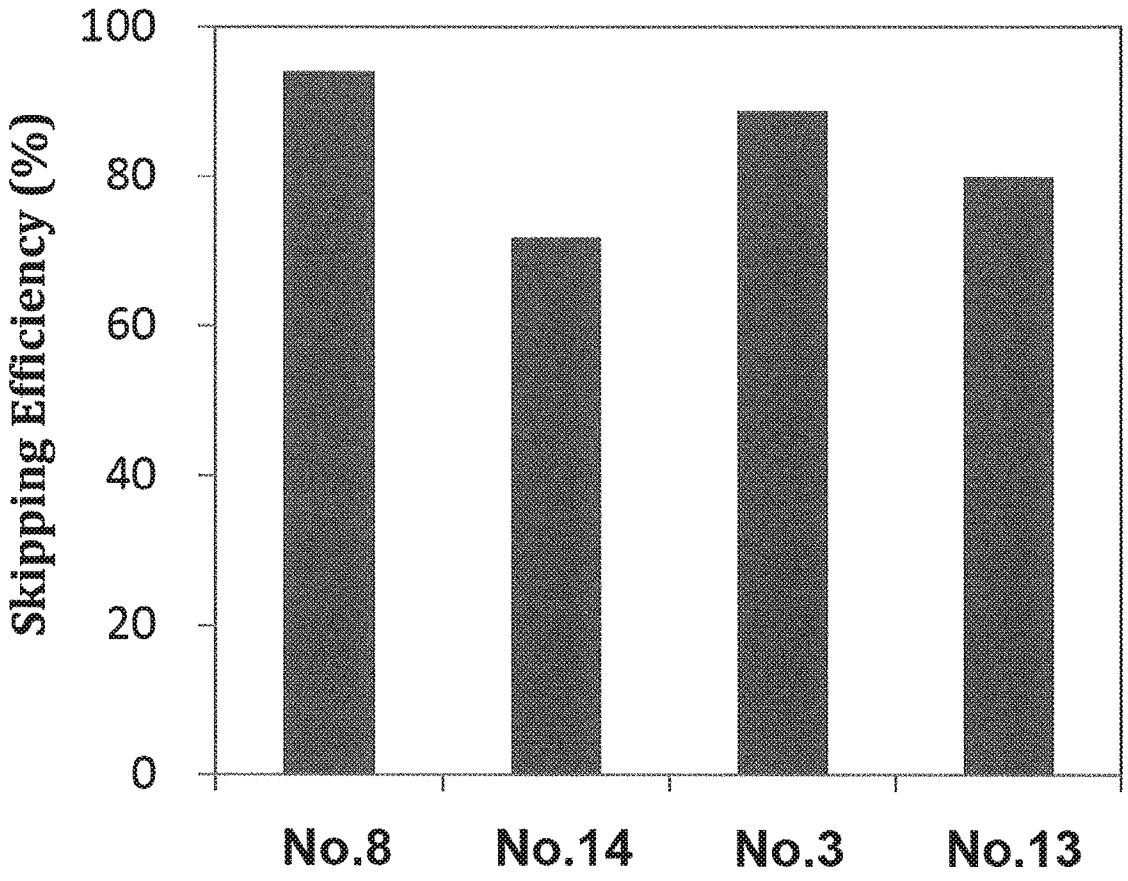

Patient with Exon 48-52 Deletion (No PMO)
Patient with Exon 45-52 Deletion (PMO No. 8)
Patient with Exon 48-52 Deletion (PMO No. 8)
Patient with Exon 48-52 Deletion (PMO No. 3)

Figure 8



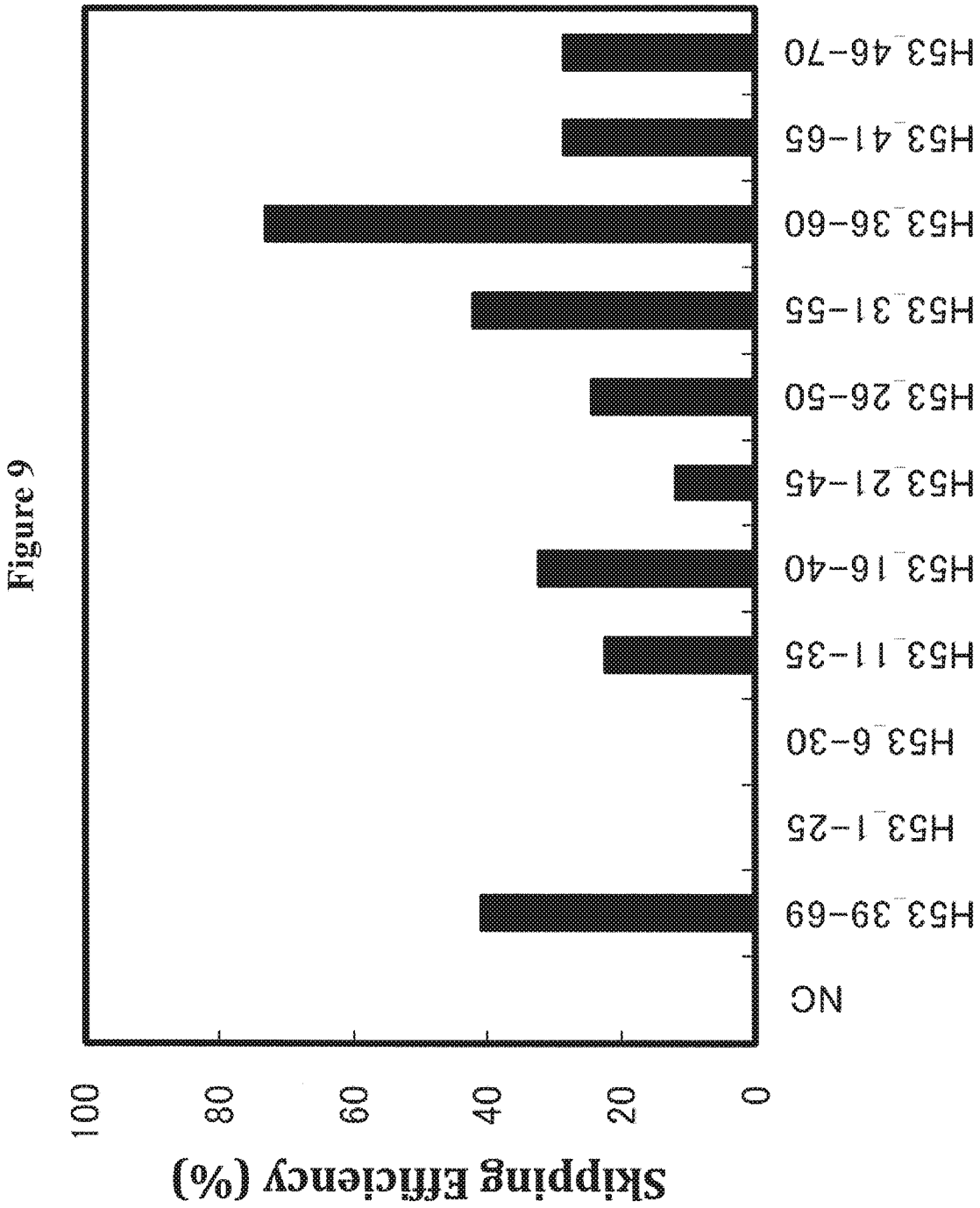

Skipping Efficiency (%)
100
80
60
40
20
0
NC
H53_39-69
H53_1-25
H53_6-30
H53_11-35
H53_16-40
H53_21-45
H53_26-50
H53_31-55
H53_36-60
H53_41-65
H53_46-70

Figure 9

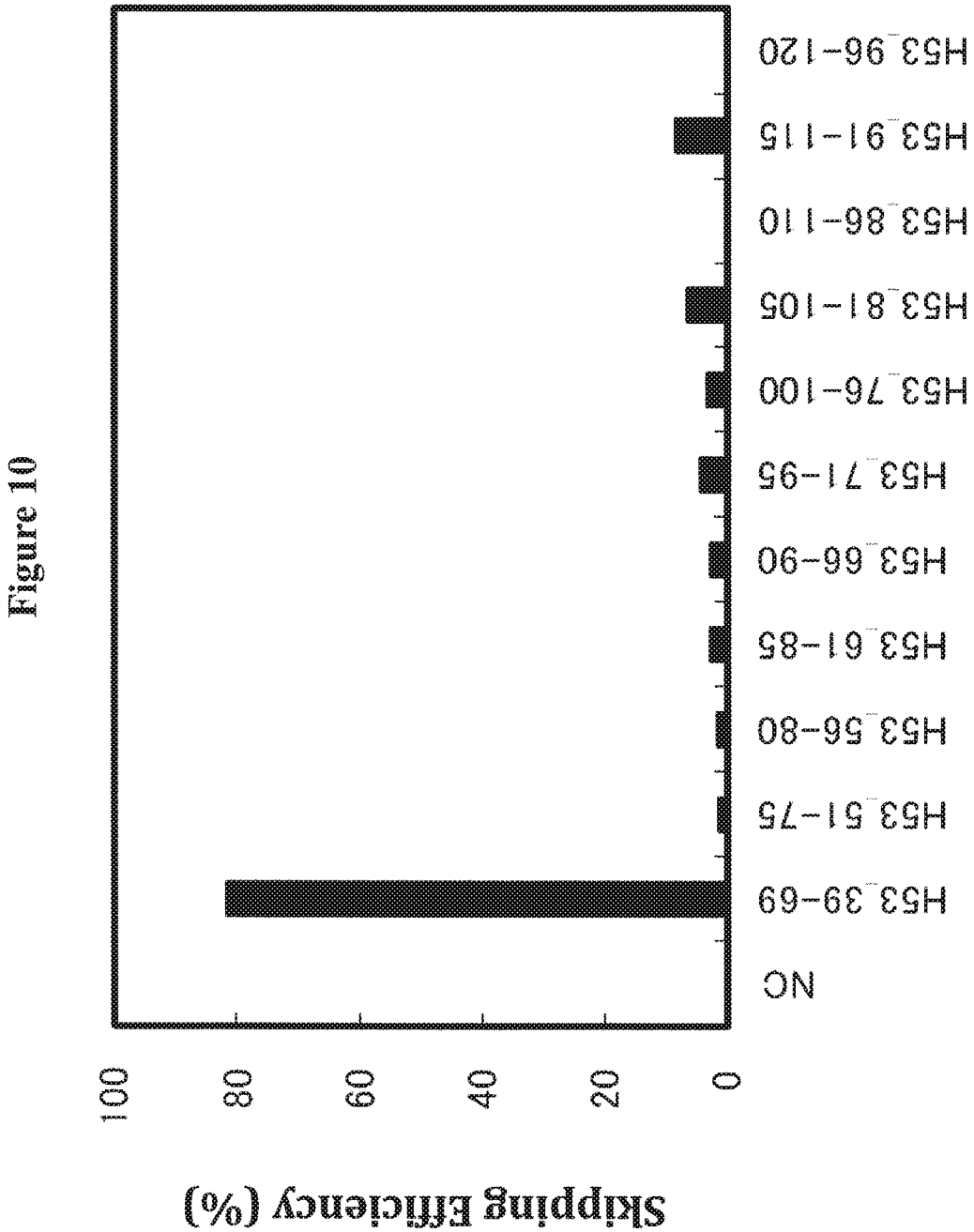

Skipping Efficiency (%)
100
80
60
40
20
0
NC
H53_39-69
H53_51-75
H53_56-80
H53_61-85
H53_66-90
H53_71-95
H53_76-100
H53_81-105
H53_86-110
H53_91-115
H53_96-120

Figure 10

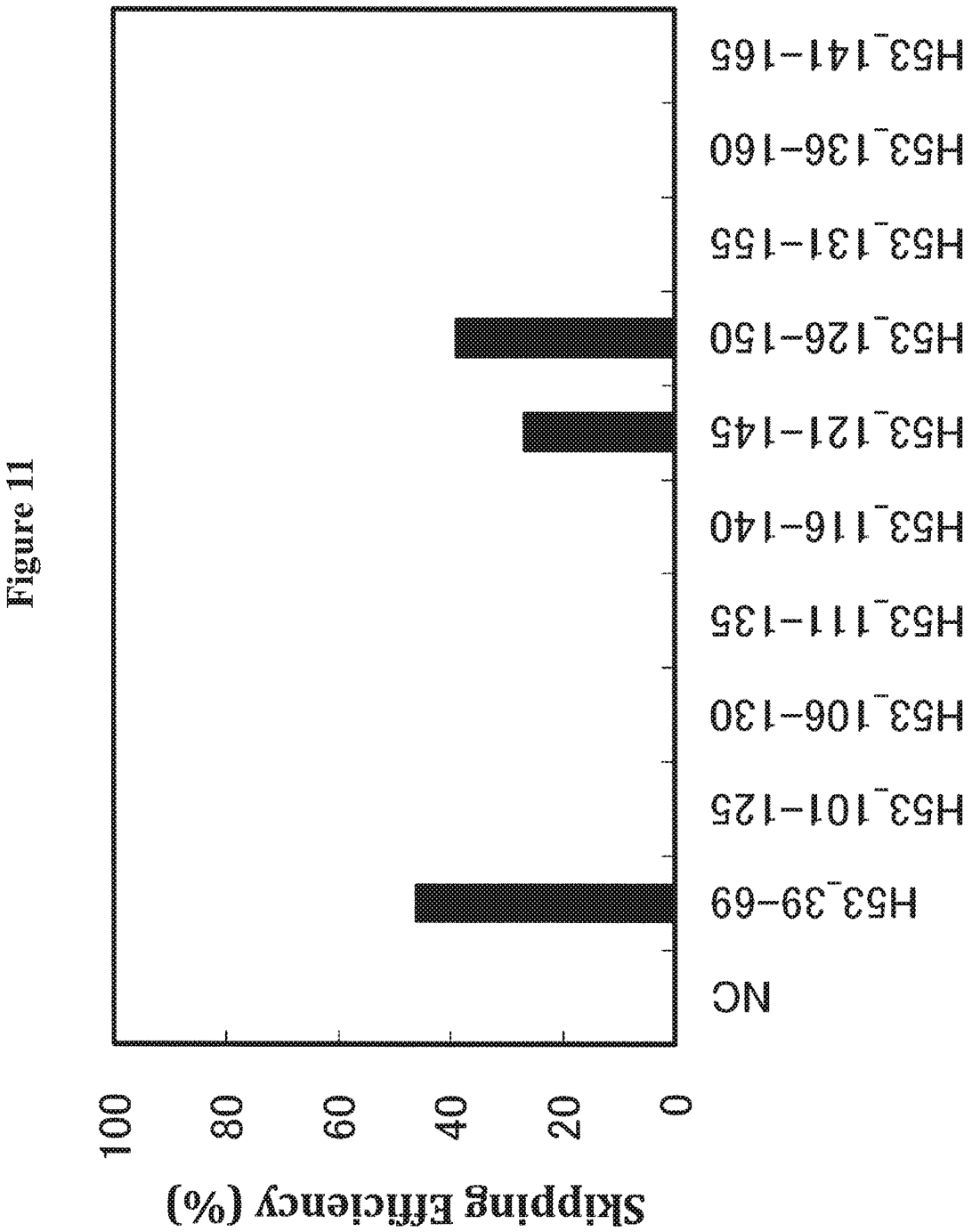

Skipping Efficiency (%)
100
80
60
40
20
0
NC
H53_39-69
H53_101-125
H53_106-130
H53_111-135
H53_116-140
H53_121-145
H53_126-150
H53_131-155
H53_136-160
H53_141-165

Figure 11

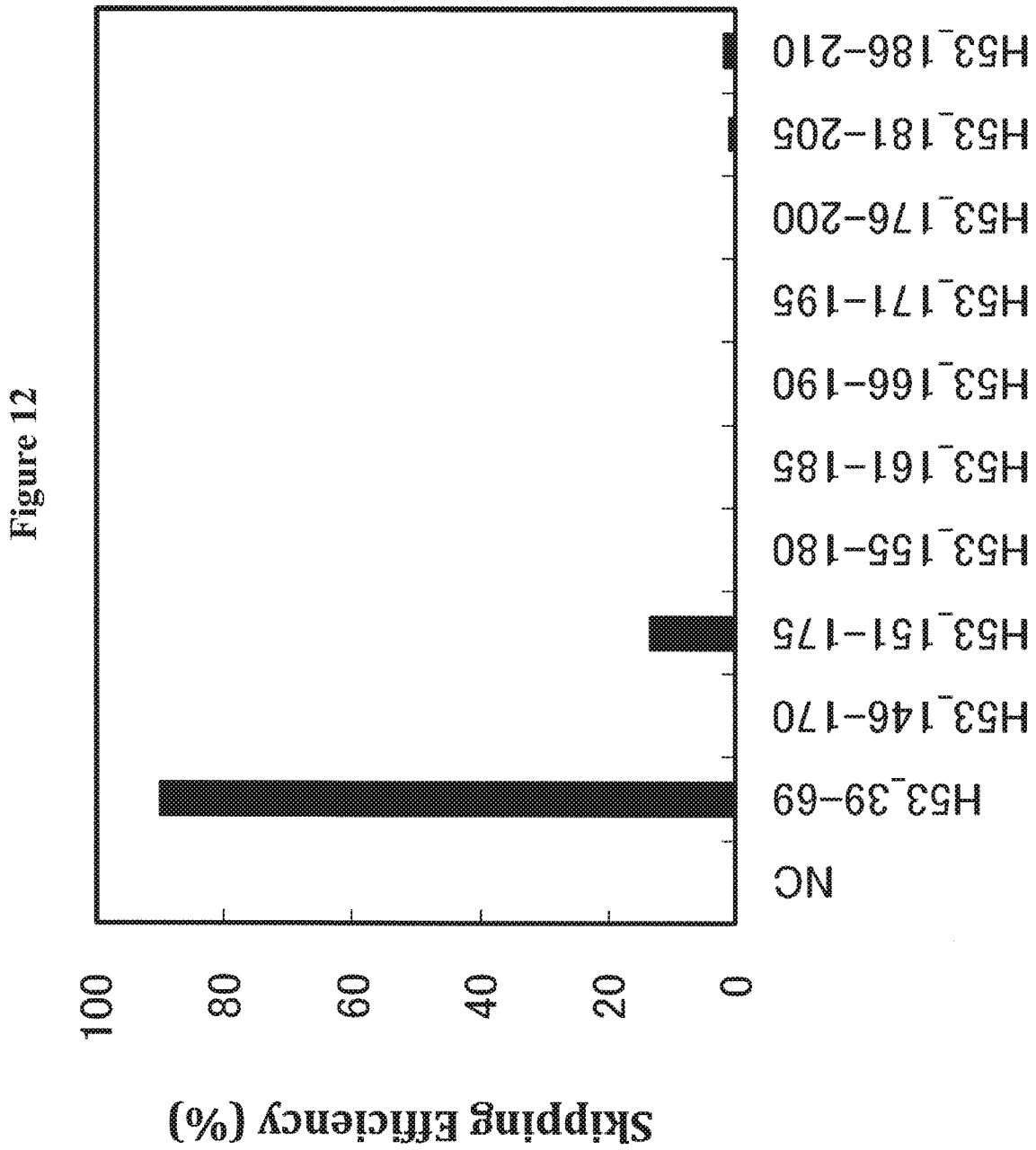

Skipping Efficiency (%)
100
80
60
40
20
0
NC
H53_39-69
H53_146-170
H53_151-175
H53_155-180
H53_161-185
H53_166-190
H53_171-195
H53_176-200
H53_181-205
H53_186-210

Figure 12

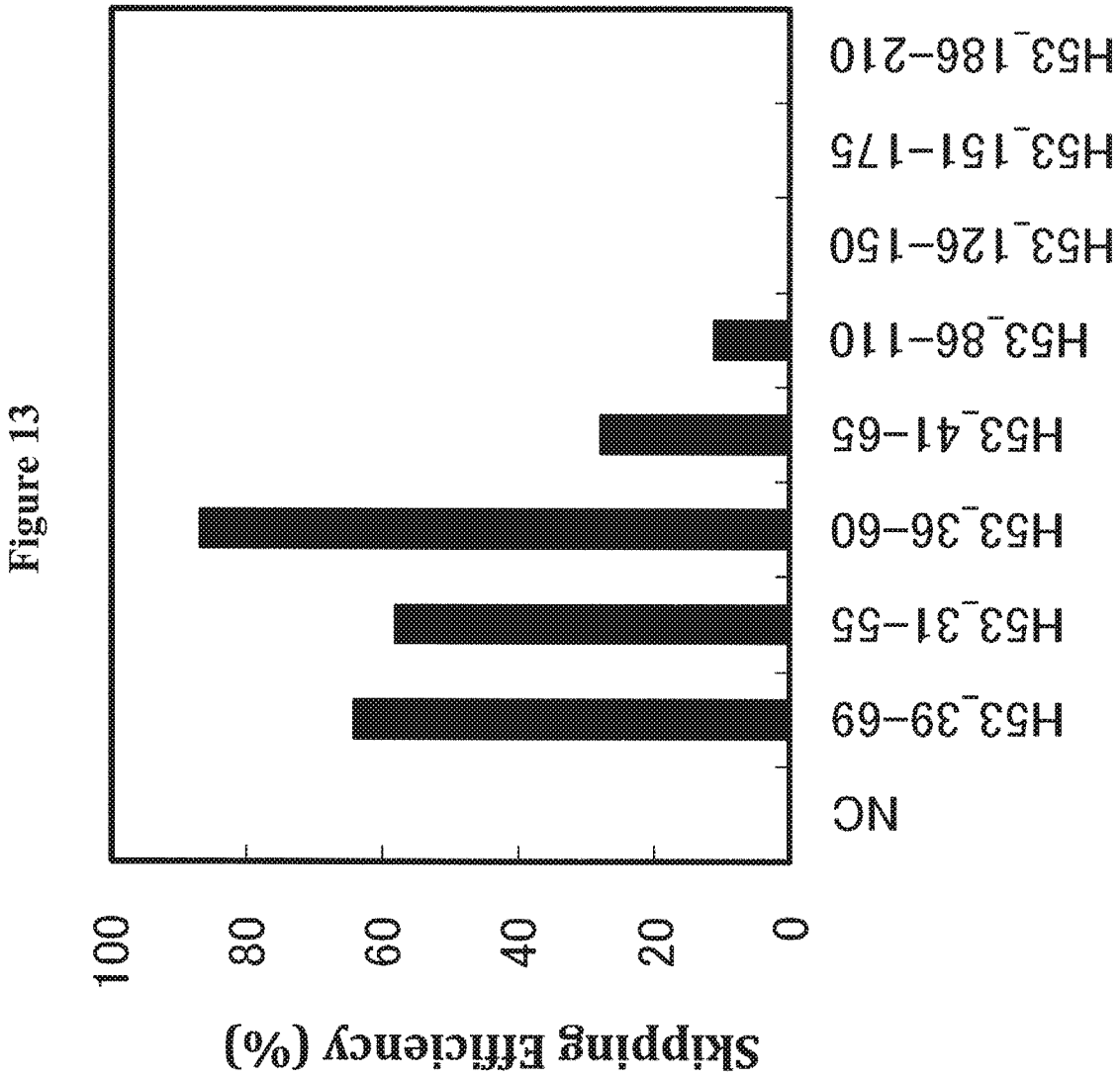

Skipping Efficiency (%)
0
20
40
60
80
100
NC
H53_39-69
H53_31-55
H53_36-60
H53_41-65
H53_86-110
H53_126-150
H53_151-175
H53_186-210

Figure 13

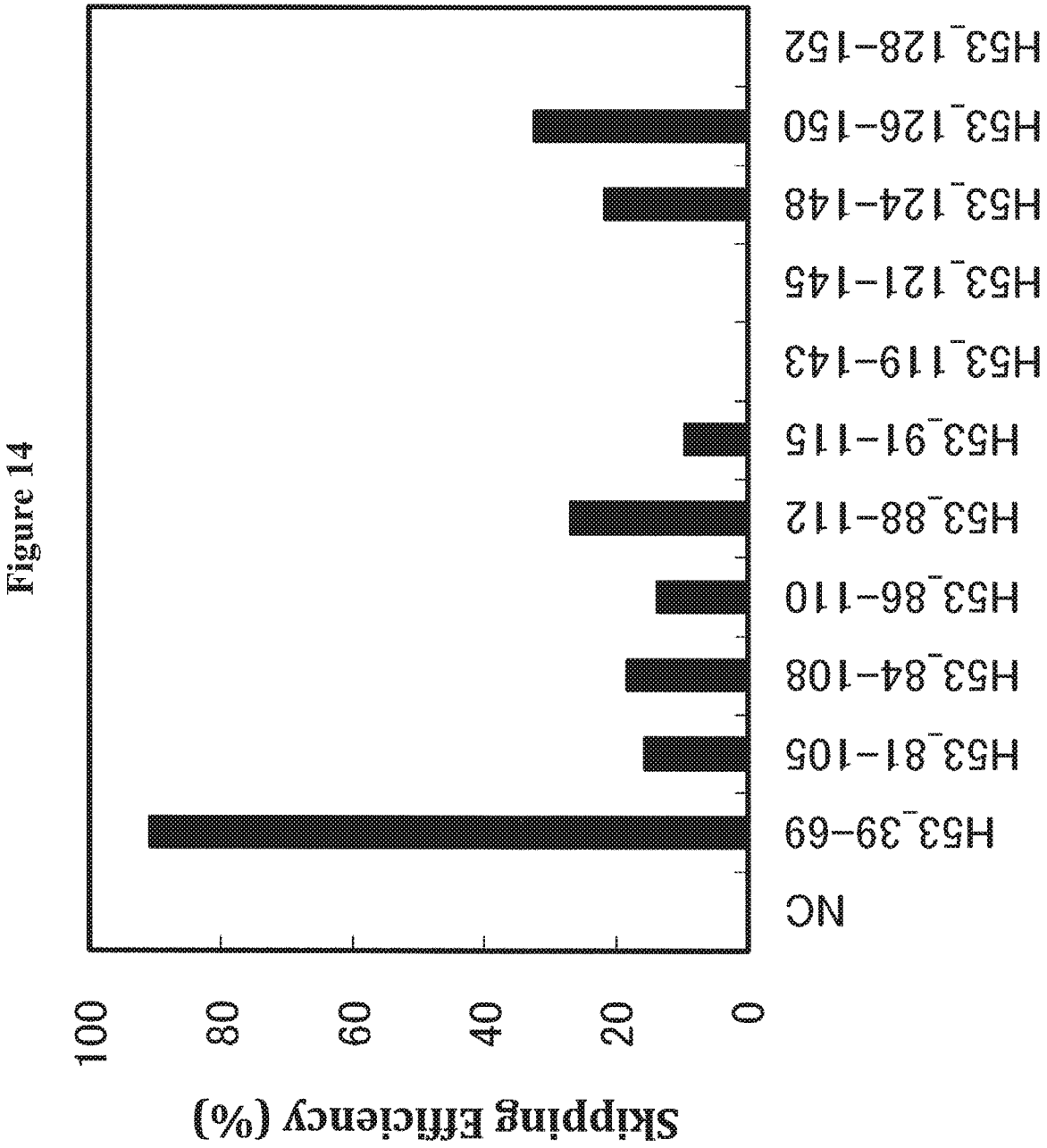

Skipping Efficiency (%)
0
20
40
60
80
100
NC
H53_39-69
H53_81-105
H53_84-108
H53_86-110
H53_88-112
H53_91-115
H53_119-143
H53_121-145
H53_124-148
H53_126-150
H53_128-152

Figure 14

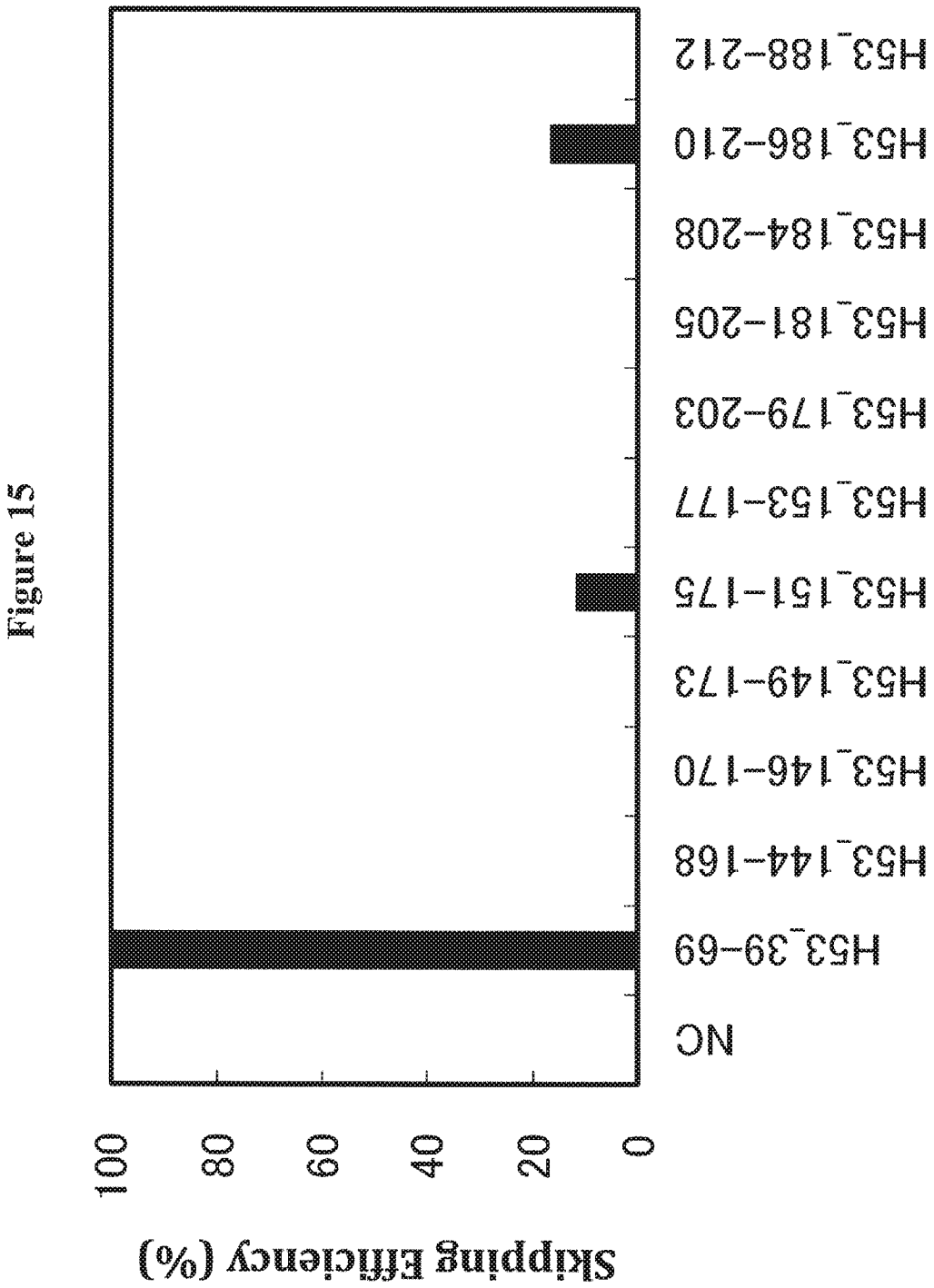

Skipping Efficiency (%)
100
80
60
40
20
0
NC
H53_39-69
H53_144-168
H53_146-170
H53_149-173
H53_151-175
H53_153-177
H53_179-203
H53_181-205
H53_184-208
H53_186-210
H53_188-212

Figure 15

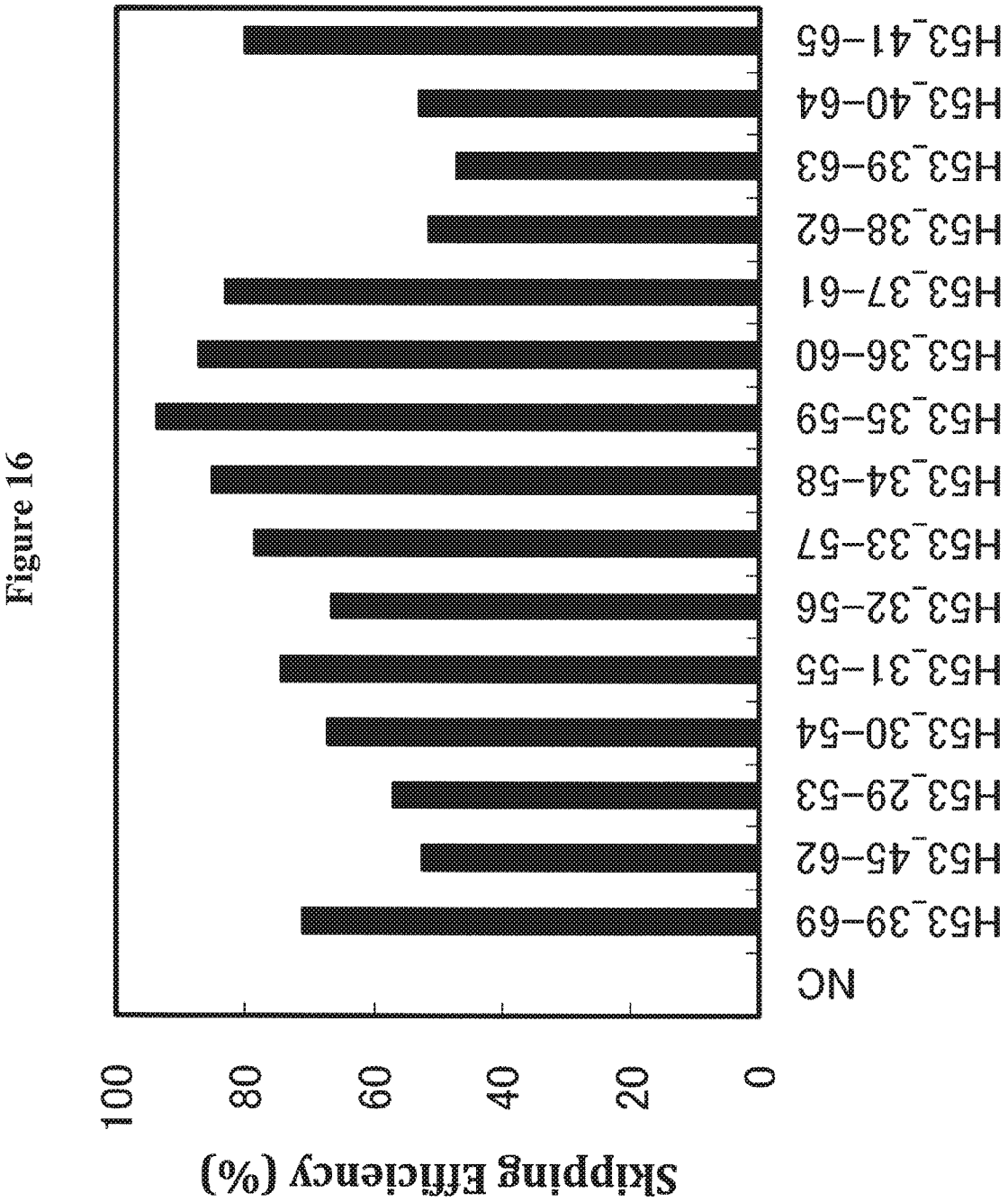

Skipping Efficiency (%)
100
80
60
40
20
0
NC
H53_39-69
H53_45-62
H53_29-53
H53_30-54
H53_31-55
H53_32-56
H53_33-57
H53_34-58
H53_35-59
H53_36-60
H53_37-61
H53_38-62
H53_39-63
H53_40-64
H53_41-65

Figure 16

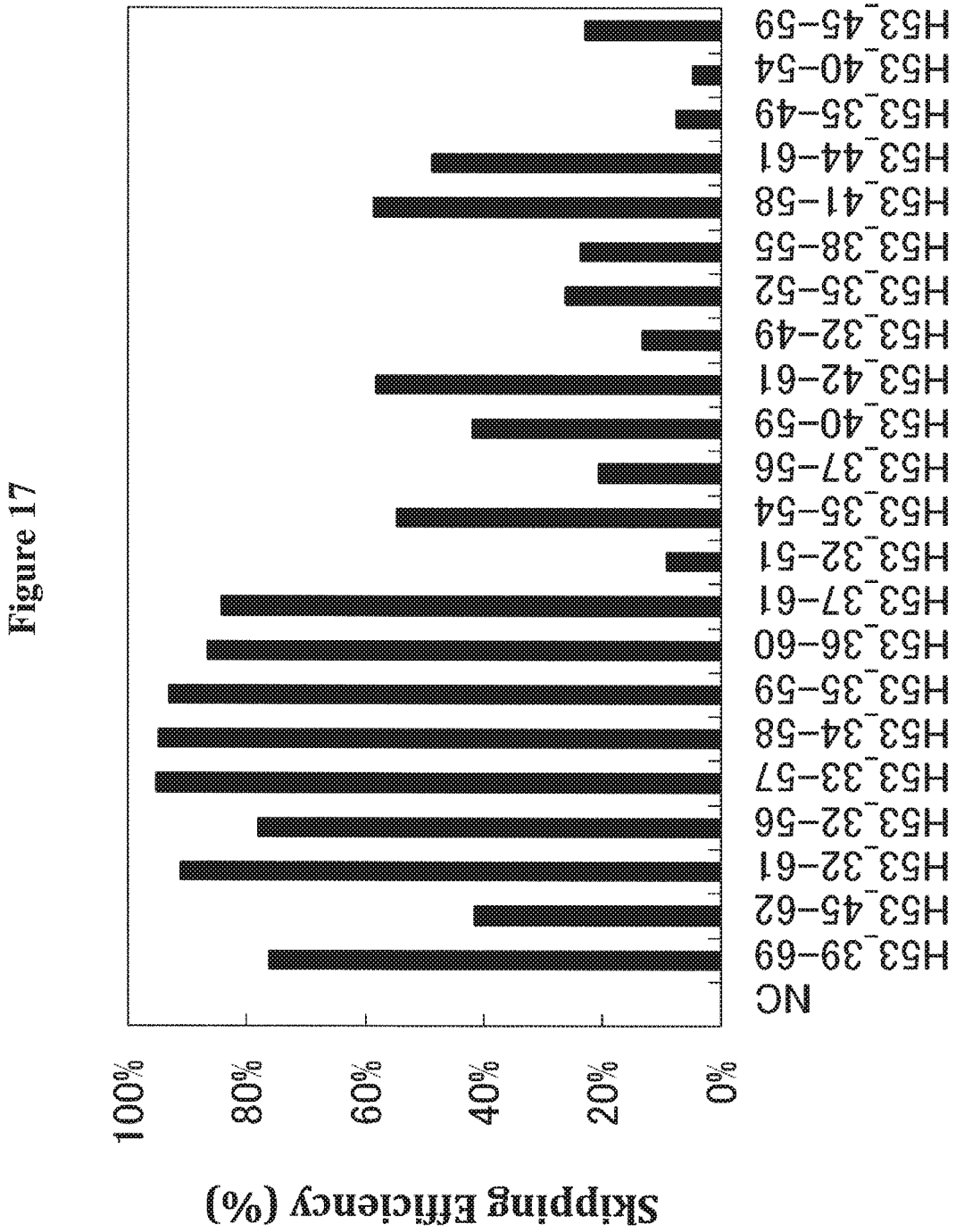

Skipping Efficiency (%)
100%
80%
60%
40%
20%
0%
NC
H53_39-69
H53_45-62
H53_32-61
H53_32-56
H53_33-57
H53_34-58
H53_35-59
H53_36-60
H53_37-61
H53_32-51
H53_35-54
H53_37-56
H53_40-59
H53_42-61
H53_32-49
H53_35-52
H53_38-55
H53_41-58
H53_44-61
H53_35-49
H53_40-54
H53_45-59

Figure 17

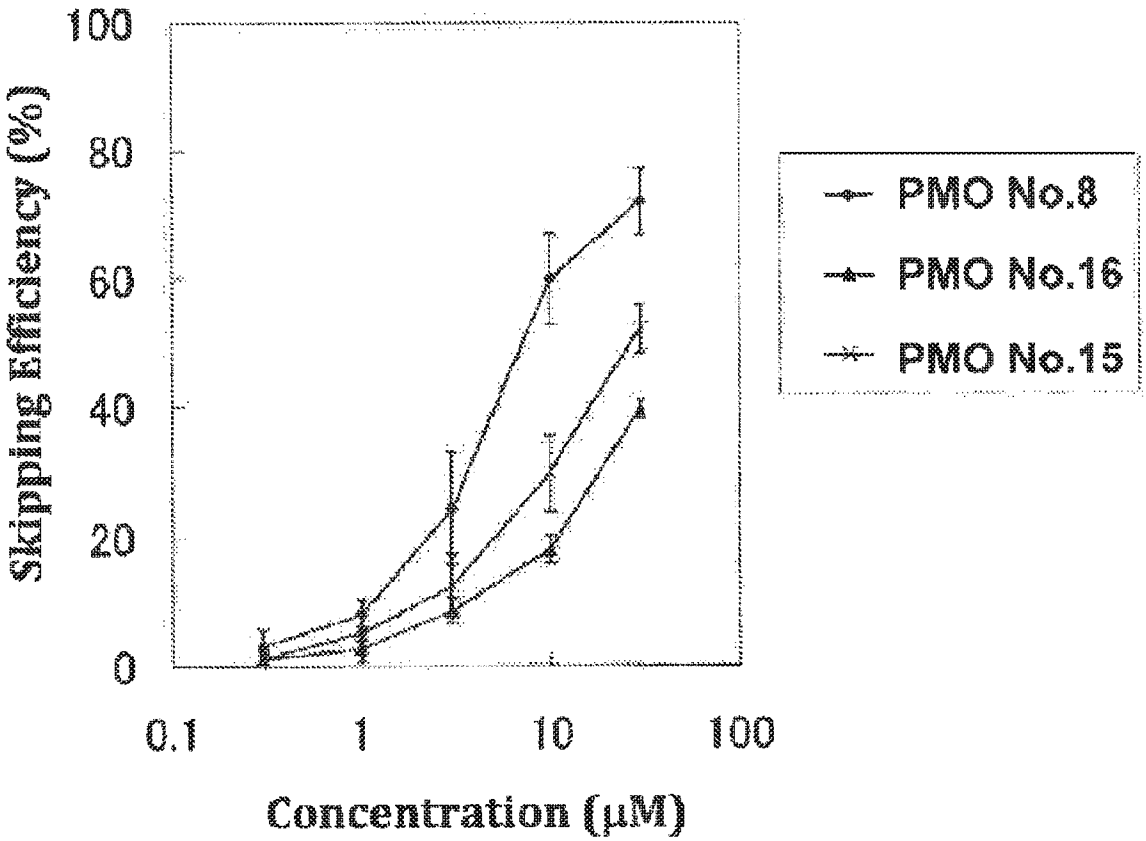

100
80
60
40
20
0
Skipping Efficiency (%)
0.1
1
10
100
Concentration (μM)
PMO No.8
PMO No.16
PMO No.15

Figure 18

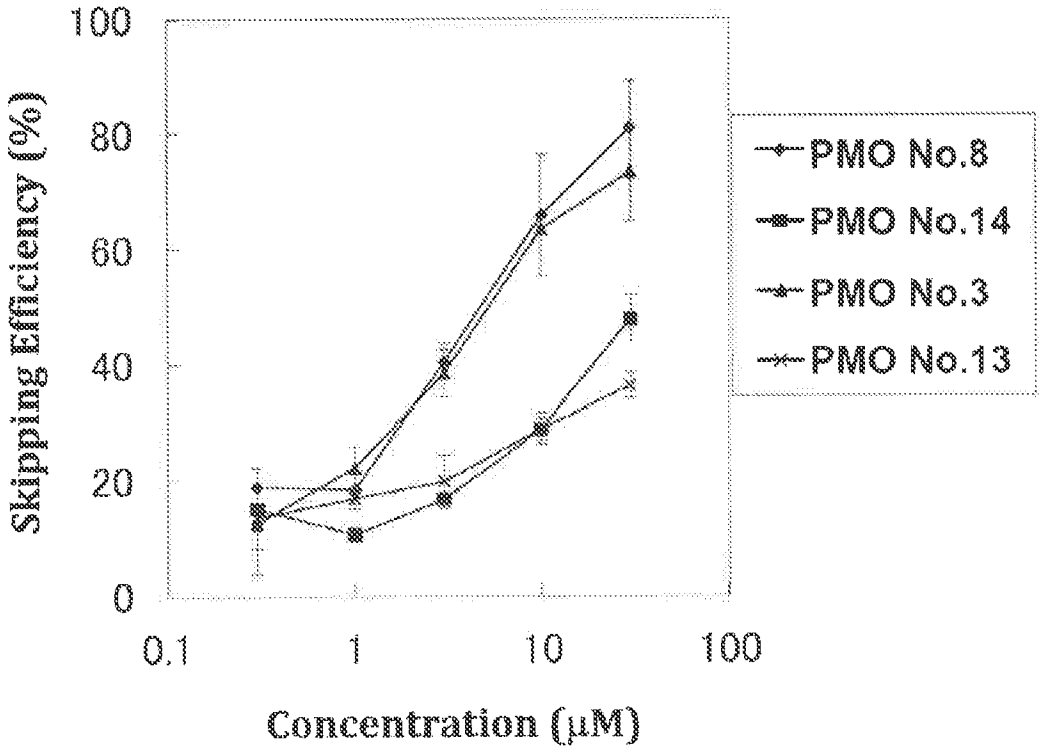

100
80
60
40
20
0
Skipping Efficiency (%)
0.1
1
10
100
Concentration (μM)
PMO No.8
PMO No.14
PMO No.3
PMO No.13

Figure 19

ANTISENSE NUCLEIC ACIDS

CROSS REFERENCE TO RELATED APPLICATIONS

This application is the National Stage of International Application No. PCT/JP2011/070318, filed Aug. 31, 2011, and claims benefit of Japanese Application No. 2010-196032, filed on Sep. 1, 2010, all of which are herein incorporated by reference in their entirety.

SEQUENCE LISTING

The instant application contains a sequence listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on Mar. 29, 2013, is named G12__0074_Seq_Listing_revised_Sq_No__64.txt and is 24,294 bytes in size.

TECHNICAL FIELD

The present invention relates to an antisense oligomer which causes skipping of exon 53 in the human dystrophin gene, and a pharmaceutical composition comprising the oligomer.

BACKGROUND ART

Duchenne muscular dystrophy (DMD) is the most frequent form of hereditary progressive muscular dystrophy that affects one in about 3,500 newborn boys. Although the motor functions are rarely different from healthy humans in infancy and childhood, muscle weakness is observed in children from around 4 to 5 years old. Then, muscle weakness progresses to the loss of ambulation by about 12 years old and death due to cardiac or respiratory insufficiency in the twenties. DMD is such a severe disorder. At present, there is no effective therapy for DMD available, and it has been strongly desired to develop a novel therapeutic agent.

DMD is known to be caused by a mutation in the dystrophin gene. The dystrophin gene is located on X chromosome and is a huge gene consisting of 2.2 million DNA nucleotide pairs. DNA is transcribed into mRNA precursors, and introns are removed by splicing to synthesize mRNA in which 79 exons are joined together. This mRNA is translated into 3,685 amino acids to produce the dystrophin protein. The dystrophin protein is associated with the maintenance of membrane stability in muscle cells and necessary to make muscle cells less fragile. The dystrophin gene from patients with DMD contains a mutation and hence, the dystrophin protein, which is functional in muscle cells, is rarely expressed. Therefore, the structure of muscle cells cannot be maintained in the body of the patients with DMD, leading to a large influx of calcium ions into muscle cells. Consequently, an inflammation-like response occurs to promote fibrosis so that muscle cells can be regenerated only with difficulty.

Becker muscular dystrophy (BMD) is also caused by a mutation in the dystrophin gene. The symptoms involve muscle weakness accompanied by atrophy of muscle but are typically mild and slow in the progress of muscle weakness, when compared to DMD. In many cases, its onset is in adulthood. Differences in clinical symptoms between DMD and BMD are considered to reside in whether the reading frame for amino acids on the translation of dystrophin mRNA into the dystrophin protein is disrupted by the mutation or not (Non-Patent Document 1). More specifically, in DMD, the presence of mutation shifts the amino acid reading frame so that the expression of functional dystrophin protein is abolished, whereas in BMD the dystrophin protein that functions, though imperfectly, is produced because the amino acid reading frame is preserved, while a part of the exons are deleted by the mutation.

Exon skipping is expected to serve as a method for treating DMD. This method involves modifying splicing to restore the amino acid reading frame of dystrophin mRNA and induce expression of the dystrophin protein having the function partially restored (Non-Patent Document 2). The amino acid sequence part, which is a target for exon skipping, will be lost. For this reason, the dystrophin protein expressed by this treatment becomes shorter than normal one but since the amino acid reading frame is maintained, the function to stabilize muscle cells is partially retained. Consequently, it is expected that exon skipping will lead DMD to the similar symptoms to that of BMD which is milder. The exon skipping approach has passed the animal tests using mice or dogs and now is currently assessed in clinical trials on human DMD patients.

The skipping of an exon can be induced by binding of antisense nucleic acids targeting either 5' or 3' splice site or both sites, or exon-internal sites. An exon will only be included in the mRNA when both splice sites thereof are recognized by the spliceosome complex. Thus, exon skipping can be induced by targeting the splice sites with antisense nucleic acids. Furthermore, the binding of an SR protein to an exonic splicing enhancer (ESE) is considered necessary for an exon to be recognized by the splicing mechanism. Accordingly, exon skipping can also be induced by targeting ESE.

Since a mutation of the dystrophin gene may vary depending on DMD patients, antisense nucleic acids need to be designed based on the site or type of respective genetic mutation. In the past, antisense nucleic acids that induce exon skipping for all 79 exons were produced by Steve Wilton, et al., University of Western Australia (Non-Patent Document 3), and the antisense nucleic acids which induce exon skipping for 39 exons were produced by Annemieke Aartsma-Rus, et al., Netherlands (Non-Patent Document 4).

It is considered that approximately 8% of all DMD patients may be treated by skipping the 53rd exon (hereinafter referred to as "exon 53"). In recent years, a plurality of research organizations reported on the studies where exon 53 in the dystrophin gene was targeted for exon skipping (Patent Documents 1 to 4; Non-Patent Document 5). However, a technique for skipping exon 53 with a high efficiency has not yet been established.

Patent Document 1: International Publication WO 2006/000057

Patent Document 2: International Publication WO 2004/048570

Patent Document 3: US 2010/0168212

Patent Document 4: International Publication WO 2010/048586

Non-Patent Document 1: Monaco A. P. et al., Genomics 1988; 2: p. 90-95

Non-Patent Document 2: Matsuo M., Brain Dev 1996; 18: p. 167-172

Non-Patent Document 3: Wilton S. D., et al., Molecular Therapy 2007: 15: p. 1288-96

Non-Patent Document 4: Annemieke Aartsma-Rus et al., (2002) Neuromuscular Disorders 12: S71-S77

Non-Patent Document 5: Linda J. Popplewell et al., (2010) Neuromuscular Disorders, vol. 20, no. 2, p. 102-10

DISCLOSURE OF THE INVENTION

Under the foregoing circumstances, antisense oligomers that strongly induce exon 53 skipping in the dystrophin gene and muscular dystrophy therapeutics comprising oligomers thereof have been desired.

As a result of detailed studies of the structure of the dystrophin gene, the present inventors have found that exon 53 skipping can be induced with a high efficiency by targeting the sequence consisting of the 32nd to the 56th nucleotides from the 5' end of exon 53 in the mRNA precursor (hereinafter referred to as "pre-mRNA") in the dystrophin gene with antisense oligomers. Based on this finding, the present inventors have accomplished the present invention.

That is, the present invention is as follows.

[1] An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences consisting of the 31st to the 53rd, the 31st to the 54th, the 31st to the 55th, the 31st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th, or the 36th to the 58th nucleotides, from the 5' end of the 53rd exon in the human dystrophin gene.

[2] The antisense oligomer according to [1] above, which is an oligonucleotide.

[3] The antisense oligomer according to [2] above, wherein the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified.

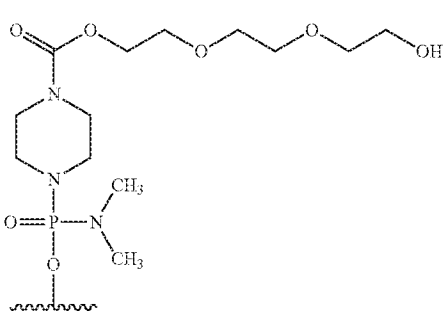
[4] The antisense oligomer according to [3] above, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br and I (wherein R is an alkyl or an aryl and R' is an alkylene).

[5] The antisense oligomer according to [3] or [4] above, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond and a boranophosphate bond.

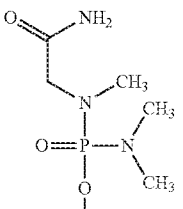
[6] The antisense oligomer according to [1] above, which is a morpholino oligomer.

[7] The antisense oligomer according to [6] above, which is a phosphorodiamidate morpholino oligomer.

[8] The antisense oligomer according to any one of [1] to [7] above, wherein the 5' end is any one of the groups of chemical formulae (1) to (3) below:



(1)



(2)

(3)

[9] The antisense oligomer according to any one of [1] to [8] above, consisting of a nucleotide sequence complementary to the sequences consisting of the 32nd to the 56th or the 36th to the 56th nucleotides from the 5' end of the 53rd exon in the human dystrophin gene.

[10] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by any one selected from the group consisting of SEQ ID NOS: 2 to 37.

[11] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by any one selected from the group consisting of SEQ ID NOS: 11, 17, 23, 29 and 35.

[12] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by SEQ ID NO: 11 or 35.

[13] A pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the antisense oligomer according to any one of [1] to [12] above, or a pharmaceutically acceptable salt or hydrate thereof.

The antisense oligomer of the present invention can induce exon 53 skipping in the human dystrophin gene with a high efficiency. In addition, the symptoms of Duchenne muscular dystrophy can be effectively alleviated by administering the pharmaceutical composition of the present invention.

BRIEF DESCRIPTION OF DRAWINGS

FIG. **1** shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cell line (RD cells).

FIG. **2** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into human normal tissue-derived fibroblasts (TIG-119 cells) to induce differentiation into muscle cells.

FIG. **3** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into human DMD patient-derived fibroblasts (5017 cells) to induce differentiation into muscle cells.

FIG. **4** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52) to induce differentiation into muscle cells.

FIG. **5** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. **6** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. **7** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52 or deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. **8** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52) to induce differentiation into muscle cells.

FIG. **9** shows the efficiency of exon 53 skipping (2'-OMe-S—RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **10** shows the efficiency of exon 53 skipping (T-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **11** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **12** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **13** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **14** shows the efficiency of exon 53 skipping (T-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **15** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **16** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **17** shows the efficiency of exon 53 skipping (T-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **18** shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at the respective concentrations of the oligomers.

FIG. **19** shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at the respective concentrations of the oligomers.

BEST MODE FOR CARRYING OUT THE INVENTION

Hereinafter, the present invention is described in detail. The embodiments described below are intended to be presented by way of example merely to describe the invention but not limited only to the following embodiments. The present invention may be implemented in various ways without departing from the gist of the invention.

All of the publications, published patent applications, patents and other patent documents cited in the specification are herein incorporated by reference in their entirety. The specification hereby incorporates by reference the contents of the specification and drawings in the Japanese Patent Application (No. 2010-196032) filed Sep. 1, 2010, from which the priority was claimed.

1. Antisense Oligomer

The present invention provides the antisense oligomer (hereinafter referred to as the "oligomer of the present invention") which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences (hereinafter also referred to as "target sequences") consisting of the 31st to the 53rd, the 31st to the 54th, the 31st to the 55th, the 31st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th, or the 36th to the 58th nucleotides, from the 5' end of the 53rd exon in the human dystrophin gene.

[Exon 53 in Human Dystrophin Gene]

In the present invention, the term "gene" is intended to mean a genomic gene and also include cDNA, mRNA precursor and mRNA. Preferably, the gene is mRNA precursor, i.e., pre-mRNA.

In the human genome, the human dystrophin gene locates at locus Xp21.2. The human dystrophin gene has a size of 3.0 Mbp and is the largest gene among known human genes. However, the coding regions of the human dystrophin gene are only 14 kb, distributed as 79 exons throughout the human dystrophin gene (Roberts, R G., et al., Genomics, 16: 536-538 (1993)). The pre-mRNA, which is the transcript of the human dystrophin gene, undergoes splicing to generate mature mRNA of 14 kb. The nucleotide sequence of human wild-type dystrophin gene is known (GenBank Accession No. NM_004006).

The nucleotide sequence of exon 53 in the human wild-type dystrophin gene is represented by SEQ ID NO: 1.

The oligomer of the present invention is designed to cause skipping of exon 53 in the human dystrophin gene, thereby modifying the protein encoded by DMD type of dystrophin gene into the BMD type of dystrophin protein. Accordingly, exon 53 in the dystrophin gene that is the target of exon skipping by the oligomer of the present invention includes both wild and mutant types.

Specifically, exon 53 mutants of the human dystrophin gene include the polynucleotides defined in (a) or (b) below.

(a) A polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1; and,

(b) A polynucleotide consisting of a nucleotide sequence having at least 90% identity with the nucleotide sequence of SEQ ID NO: 1.

As used herein, the term "polynucleotide" is intended to mean DNA or RNA.

As used herein, the term "polynucleotide that hybridizes under stringent conditions" refers to, for example, a poly-

nucleotide obtained by colony hybridization, plaque hybridization, Southern hybridization or the like, using as a probe all or part of a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of, e.g., SEQ ID NO: 1. The hybridization method which may be used includes methods described in, for example, "Sambrook & Russell, Molecular Cloning: A Laboratory Manual Vol. 3, Cold Spring Harbor, Laboratory Press 2001," "Ausubel, Current Protocols in Molecular Biology, John Wiley & Sons 1987-1997," etc.

As used herein, the term "complementary nucleotide sequence" is not limited only to nucleotide sequences that form Watson-Crick pairs with target nucleotide sequences, but is intended to also include nucleotide sequences which form Wobble base pairs. As used herein, the term Watson-Crick pair refers to a pair of nucleobases in which hydrogen bonds are formed between adenine-thymine, adenine-uracil or guanine-cytosine, and the term Wobble base pair refers to a pair of nucleobases in which hydrogen bonds are formed between guanine-uracil, inosine-uracil, inosine-adenine or inosine-cytosine. As used herein, the term "complementary nucleotide sequence" does not only refers to a nucleotide sequence 100% complementary to the target nucleotide sequence but also refers to a complementary nucleotide sequence that may contain, for example, 1 to 3, 1 or 2, or one nucleotide non-complementary to the target nucleotide sequence.

As used herein, the term "stringent conditions" may be any of low stringent conditions, moderate stringent conditions or high stringent conditions. The term "low stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 32° C. The term "moderate stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 42° C., or 5×SSC, 1% SDS, 50 mM Tris-HCl (pH 7.5), 50% formamide at 42° C. The term "high stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 50° C. or 0.2×SSC, 0.1% SDS at 65° C. Under these conditions, polynucleotides with higher homology are expected to be obtained efficiently at higher temperatures, although multiple factors are involved in hybridization stringency including temperature, probe concentration, probe length, ionic strength, time, salt concentration and others, and those skilled in the art may appropriately select these factors to achieve similar stringency.

When commercially available kits are used for hybridization, for example, an Alkphos Direct Labeling and Detection System (GE Healthcare) may be used. In this case, according to the attached protocol, after cultivation with a labeled probe overnight, the membrane is washed with a primary wash buffer containing 0.1% (w/v) SDS at 55° C., thereby detecting hybridized polynucleotides. Alternatively, in producing a probe based on the entire or part of the nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1, hybridization can be detected with a DIG Nucleic Acid Detection Kit (Roche Diagnostics) when the probe is labeled with digoxigenin (DIG) using a commercially available reagent (e.g., a PCR Labeling Mix (Roche Diagnostics), etc.).

In addition to the polynucleotides described above, other polynucleotides that can be hybridized include polynucleotides having 90% or higher, 91% or higher, 92% or higher, 93% or higher, 94% or higher, 95% or higher, 96% or higher, 97% or higher, 98% or higher, 99% or higher, 99.1% or higher, 99.2% or higher, 99.3% or higher, 99.4% or higher, 99.5% or higher, 99.6% or higher, 99.7% or higher, 99.8% or higher or 99.9% or higher identity with the polynucleotide of SEQ ID NO: 1, as calculated by homology search software BLAST using the default parameters.

The identity between nucleotide sequences may be determined using algorithm BLAST (Basic Local Alignment Search Tool) by Karlin and Altschul (Proc. Natl. Acad. Sci. USA 872264-2268, 1990; Proc. Natl. Acad. Sci. USA 90: 5873, 1993). Programs called BLASTN and BLASTX based on the BLAST algorithm have been developed (Altschul S F, et al: J. Mol. Biol. 215: 403, 1990). When a nucleotide sequence is sequenced using BLASTN, the parameters are, for example, score=100 and wordlength=12. When BLAST and Gapped BLAST programs are used, the default parameters for each program are employed.

Examples of the nucleotide sequences complementary to the sequences consisting of the 31st to the 53rd, the 31st to the 54th, the 31st to the 55th, the 31st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th and the 36th to the 58th nucleotides, from the 5' end of exon 53.

TABLE 1

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NO:
31-53	5'-CCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 2
31-54	5'-TCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 3
31-55	5'-CTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 4
31-56	5'-CCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 5
31-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 6
31-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 7
32-53	5'-CCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 8
32-54	5'-TCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 9

TABLE 1 -continued

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NO:
32-55	5'-CTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 10
32-56	5'-CCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 11
32-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 12
32-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 13
33-53	5'-CCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 14
33-54	5'-TCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 15
33-55	5'-CTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 16
33-56	5'-CCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 17
33-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 18
33-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 19
34-53	5'-CCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 20
34-54	5'-TCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 21
34-55	5'-CTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 22
34-56	5'-CCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 23
34-57	5'-GCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 24
34-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 25
35-53	5'-CCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 26
35-54	5'-TCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 27
35-55	5'-CTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 28
35-56	5'-CCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 29
35-57	5'-GCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 30
35-58	5'-TGCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 31
36-53	5'-CCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 32
36-54	5'-TCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 33
36-55	5'-CTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 34
36-56	5'-CCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 35
36-57	5'-GCCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 36
36-58	5'-TGCCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 37

It is preferred that the oligomer of the present invention consists of a nucleotide sequence complementary to any one of the sequences consisting of the 32nd to the 56th, the 33rd to the 56th, the 34th to the 56th, the 35th to the 56th or the 36th to the 56th nucleotides (e.g., SEQ ID NO: 11, SEQ ID NO: 17, SEQ ID NO: 23, SEQ ID NO: 29 or SEQ ID NO: 35), from the 5' end of the 53rd exon in the human dystrophin gene.

Preferably, the oligomer of the present invention consists of a nucleotide sequence complementary to any one of the sequences consisting of the 32nd to the 56th or the 36th to the 56th nucleotides (e.g., SEQ ID NO: 11 or SEQ ID NO: 35), from the 5' end of the 53rd exon in the human dystrophin gene.

The term "cause skipping of the 53rd exon in the human dystrophin gene" is intended to mean that by binding of the oligomer of the present invention to the site corresponding to exon 53 of the transcript (e.g., pre-mRNA) of the human dystrophin gene, for example, the nucleotide sequence corresponding to the 5' end of exon 54 is spliced at the 3' side of the nucleotide sequence corresponding to the 3' end of exon 51 in DMD patients with deletion of, exon 52 when the transcript undergoes splicing, thus resulting in formation of mature mRNA which is free of codon frame shift.

Accordingly, it is not required for the oligomer of the present invention to have a nucleotide sequence 100% complementary to the target sequence, as far as it causes exon 53 skipping in the human dystrophin gene. The oligomer of the present invention may include, for example, 1 to 3, 1 or 2, or one nucleotide non-complementary to the target sequence.

Herein, the term "binding" described above is intended to mean that when the oligomer of the present invention is mixed

with the transcript of human dystrophin gene, both are hybridized under physiological conditions to form a double strand nucleic acid. The term "under physiological conditions" refers to conditions set to mimic the in vivo environment in terms of pH, salt composition and temperature. The conditions are, for example, 25 to 40° C., preferably 37° C., pH 5 to 8, preferably pH 7.4 and 150 mM of sodium chloride concentration.

Whether the skipping of exon 53 in the human dystrophin gene is caused or not can be confirmed by introducing the oligomer of the present invention into a dystrophin expression cell (e.g., human rhabdomyosarcoma cells), amplifying the region surrounding exon 53 of mRNA of the human dystrophin gene from the total RNA of the dystrophin expression cell by RT-PCR and performing nested PCR or sequence analysis on the PCR amplified product.

The skipping efficiency can be determined as follows. The mRNA for the human dystrophin gene is collected from test cells; in the mRNA, the polynucleotide level "A" of the band where exon 53 is skipped and the polynucleotide level "B" of the band where exon 53 is not skipped are measured. Using these measurement values of "A" and "B," the efficiency is calculated by the following equation:

$$\text{Skipping efficiency (\%)}=A/(A+B)\times 100$$

The oligomer of the present invention includes, for example, an oligonucleotide, morpholino oligomer or peptide nucleic acid (PNA), having a length of 18 to 28 nucleotides. The length is preferably from 21 to 25 nucleotides and morpholino oligomers are preferred.

The oligonucleotide described above (hereinafter referred to as "the oligonucleotide of the present invention") is the oligomer of the present invention composed of nucleotides as constituent units. Such nucleotides may be any of ribonucleotides, deoxyribonucleotides and modified nucleotides.

The modified nucleotide refers to one having fully or partly modified nucleobases, sugar moieties and/or phosphate-binding regions, which constitute the ribonucleotide or deoxyribonucleotide.

The nucleobase includes, for example, adenine, guanine, hypoxanthine, cytosine, thymine, uracil, and modified bases thereof. Examples of such modified nucleobases include, but not limited to, pseudouracil, 3-methyluracil, dihydrouracil, 5-alkylcytosines (e.g., 5-methylcytosine), 5-alkyluracils (e.g., 5-ethyluracil), 5-halouracils (5-bromouracil), 6-azapyrimidine, 6-alkylpyrimidines (6-methyluracil), 2-thiouracil, 4-thiouracil, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5'-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, 1-methyladenine, 1-methylhypoxanthine, 2,2-dimethylguanine, 3-methylcytosine, 2-methyladenine, 2-methylguanine, N6-methyladenine, 7-methylguanine, 5-methoxyaminomethyl-2-thiouracil, 5-methylaminomethyluracil, 5-methylcarbonylmethyluracil, 5-methyloxyuracil, 5-methyl-2-thiouracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid, 2-thiocytosine, purine, 2,6-diaminopurine, 2-aminopurine, isoguanine, indole, imidazole, xanthine, etc.

Modification of the sugar moiety may include, for example, modifications at the 2'-position of ribose and modifications of the other positions of the sugar. The modification at the 2'-position of ribose includes replacement of the 2'-OH of ribose with OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br or I, wherein R represents an alkyl or an aryl and R' represents an alkylene.

The modification for the other positions of the sugar includes, for example, replacement of O at the 4' position of ribose or deoxyribose with S, bridging between 2' and 4' positions of the sugar, e.g., LNA (locked nucleic acid) or ENA (2'-O,4'-C-ethylene-bridged nucleic acids), but is not limited thereto.

A modification of the phosphate-binding region includes, for example, a modification of replacing phosphodiester bond with phosphorothioate bond, phosphorodithioate bond, alkyl phosphonate bond, phosphoroamidate bond or boranophosphate bond (Enya et al: Bioorganic & Medicinal Chemistry, 2008, 18, 9154-9160) (cf., e.g., Japan Domestic Re-Publications of PCT Application Nos. 2006/129594 and 2006/038608).

The alkyl is preferably a straight or branched alkyl having 1 to 6 carbon atoms. Specific examples include methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, n-pentyl, isopentyl, neopentyl, tert-pentyl, n-hexyl and isohexyl. The alkyl may optionally be substituted. Examples of such substituents are a halogen, an alkoxy, cyano and nitro. The alkyl may be substituted with 1 to 3 substituents.

The cycloalkyl is preferably a cycloalkyl having 5 to 12 carbon atoms. Specific examples include cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, cyclodecyl and cyclododecyl.

The halogen includes fluorine, chlorine, bromine and iodine.

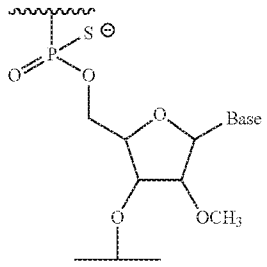
The alkoxy is a straight or branched alkoxy having 1 to 6 carbon atoms such as methoxy, ethoxy, n-propoxy, isopropoxy, n-butoxy, isobutoxy, sec-butoxy, tert-butoxy, n-pentyloxy, isopentyloxy, n-hexyloxy, isohexyloxy, etc. Among others, an alkoxy having 1 to 3 carbon atoms is preferred.

The aryl is preferably an aryl having 6 to 10 carbon atoms. Specific examples include phenyl, α-naphthyl and β-naphthyl. Among others, phenyl is preferred. The aryl may optionally be substituted. Examples of such substituents are an alkyl, a halogen, an alkoxy, cyano and nitro. The aryl may be substituted with one to three of such substituents.

The alkylene is preferably a straight or branched alkylene having 1 to 6 carbon atoms. Specific examples include methylene, ethylene, trimethylene, tetramethylene, pentamethylene, hexamethylene, 2-(ethyl) trimethylene and 1-(methyl) tetramethylene.

The acyl includes a straight or branched alkanoyl or aroyl. Examples of the alkanoyl include formyl, acetyl, 2-methylacetyl, 2,2-dimethylacetyl, propionyl, butyryl, isobutyryl, pentanoyl, 2,2-dimethylpropionyl, hexanoyl, etc. Examples of the aroyl include benzoyl, toluoyl and naphthoyl. The aroyl may optionally be substituted at substitutable positions and may be substituted with an alkyl(s).

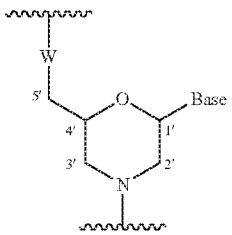
Preferably, the oligonucleotide of the present invention is the oligomer of the present invention containing a constituent unit represented by general formula below wherein the —OH group at position 2' of ribose is substituted with methoxy and the phosphate-binding region is a phosphorothioate bond:



wherein Base represents a nucleobase.

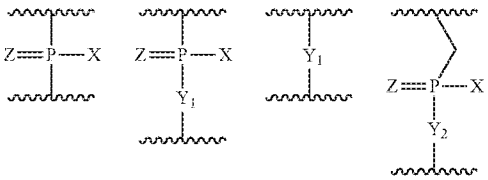
The oligonucleotide of the present invention may be easily synthesized using various automated synthesizer (e.g., AKTA oligopilot plus 10/100 (GE Healthcare)). Alternatively, the synthesis may also be entrusted to a third-party organization (e.g., Promega Inc., or Takara Co.), etc.

The morpholino oligomer of the present invention is the oligomer of the present invention comprising the constituent unit represented by general formula below:



wherein Base has the same significance as defined above, and,

W represents a group shown by any one of the following groups:



wherein X represents —CH_2R^1, —O—CH_2R^1, —S—CH_2R^1, —NR_2R^3 or F;

R^1 represents H or an alkyl;

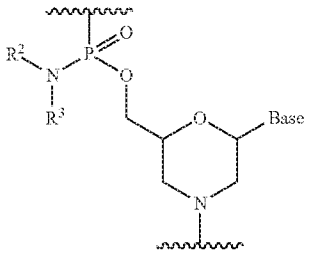
R^2 and R^3, which may be the same or different, each represents H, an alkyl, a cycloalkyl or an aryl;

Y_1 represents O, S, CH_2 or NR^1;

Y_2 represents O, S or NR^1;

Z represents O or S.

Preferably, the morpholino oligomer is an oligomer comprising a constituent unit represented by general formula below (phosphorodiamidate morpholino oligomer (hereinafter referred to as "PMO")).

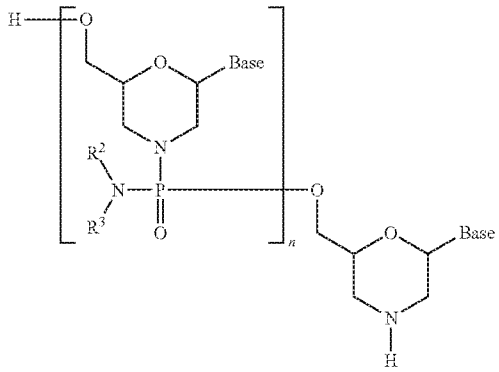


wherein Base, R^2 and R^3 have the same significance as defined above.

The morpholino oligomer may be produced in accordance with, e.g., WO 1991/009033 or WO 2009/064471. In particular, PMO can be produced by the procedure described in WO 2009/064471 or produced by the process shown below.

[Method for Producing PMO]

An embodiment of PMO is, for example, the compound represented by general formula (I) below (hereinafter PMO (I)).



(I)

wherein Base, R^2 and R^3 have the same significance as defined above; and,

n is a given integer of 1 to 99, preferably a given integer of 18 to 28.

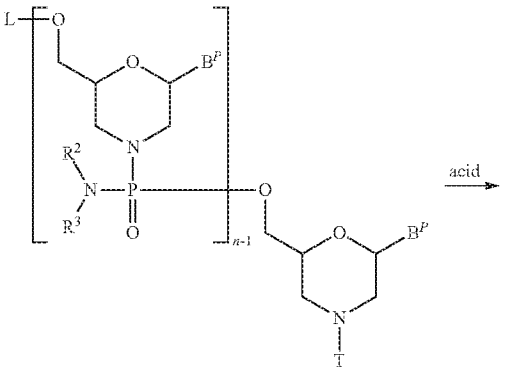
PMO (I) can be produced in accordance with a known method, for example, can be produced by performing the procedures in the following steps.

The compounds and reagents used in the steps below are not particularly limited so long as they are commonly used to prepare PMO.

Also, the following steps can all be carried out by the liquid phase method or the solid phase method (using manuals or commercially available solid phase automated synthesizers). In producing PMO by the solid phase method, it is desired to use automated synthesizers in view of simple operation procedures and accurate synthesis.

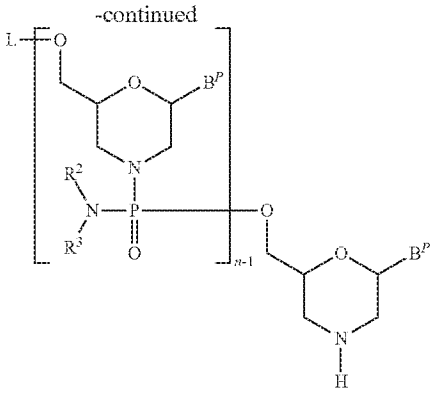
(1) Step A:

The compound represented by general formula (II) below (hereinafter referred to as Compound (II)) is reacted with an acid to prepare the compound represented by general formula (III) below (hereinafter referred to as Compound (III)):



(II)

-continued



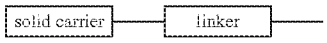
(III)

wherein n, R^2 and R^3 have the same significance as defined above;

each B^P independently represents a nucleobase which may optionally be protected;

T represents trityl, monomethoxytrityl or dimethoxytrityl; and,

L represents hydrogen, an acyl or a group represented by general formula (IV) below (hereinafter referred to as group (IV)).



(IV)

The "nucleobase" for B^P includes the same "nucleobase" as in Base, provided that the amino or hydroxy group in the nucleobase shown by B^P may be protected.

Such protective group for amino is not particularly limited so long as it is used as a protective group for nucleic acids. Specific examples include benzoyl, 4-methoxybenzoyl, acetyl, propionyl, butyryl, isobutyryl, phenylacetyl, phenoxyacetyl, 4-tert-butylphenoxyacetyl, 4-isopropylphenoxyacetyl and (dimethylamino)methylene. Specific examples of the protective group for the hydroxy group include 2-cyanoethyl, 4-nitrophenethyl, phenylsulfonylethyl, methylsulfonylethyl and trimethylsilylethyl, and phenyl, which may be substituted by 1 to 5 electron-withdrawing group at optional substitutable positions, diphenylcarbamoyl, dimethylcarbamoyl, diethylcarbamoyl, methylphenylcarbamoyl, 1-pyrrolidinylcarbamoyl, morpholinocarbamoyl, 4-(tert-butylcarboxy) benzyl, 4-[(dimethylamino)carboxyl]benzyl and 4-(phenylcarboxy)benzyl, (cf., e.g., WO 2009/064471).

The "solid carrier" is not particularly limited so long as it is a carrier usable for the solid phase reaction of nucleic acids. It is desired for the solid carrier to have the following properties: e.g., (i) it is sparingly soluble in reagents that can be used for the synthesis of morpholino nucleic acid derivatives (e.g., dichloromethane, acetonitrile, tetrazole, N-methylimidazole, pyridine, acetic anhydride, lutidine, trifluoroacetic acid); (ii) it is chemically stable to the reagents usable for the synthesis of morpholino nucleic acid derivatives; (iii) it can be chemically modified; (iv) it can be charged with desired morpholino nucleic acid derivatives; (v) it has a strength sufficient to withstand high pressure through treatments; and (vi) it has a uniform particle diameter range and distribution. Specifically, swellable polystyrene (e.g., aminomethyl polystyrene resin 1% dibenzylbenzene crosslinked (200-400 mesh) (2.4-3.0 mmol/g) (manufactured by Tokyo Chemical Industry), Aminomethylated Polystyrene Resin.HCl [dibenzylbenzene 1%, 100-200 mesh] (manufactured by Peptide Institute, Inc.)), non-swellable polystyrene (e.g., Primer Support (manufactured by GE Healthcare)), PEG chain-attached polystyrene (e.g., NH_2—PEG resin (manufactured by Watanabe Chemical Co.), TentaGel resin), controlled pore glass (controlled pore glass; CPG) (manufactured by, e.g., CPG), oxalyl-controlled pore glass (cf., e.g., Alul et al., Nucleic Acids Research, Vol. 19, 1527 (1991)), TentaGel support-aminopolyethylene glycol-derivatized support (e.g., Wright et al., cf., Tetrahedron Letters, Vol. 34, 3373 (1993)), and a copolymer of Poros-polystyrene/divinylbenzene.

A "linker" which can be used is a known linker generally used to connect nucleic acids or morpholino nucleic acid derivatives. Examples include 3-aminopropyl, succinyl, 2,2'-diethanolsulfonyl and a long chain alkyl amino (LCAA).

This step can be performed by reacting Compound (II) with an acid.

The "acid" which can be used in this step includes, for example, trifluoroacetic acid, dichloroacetic acid and trichloroacetic acid. The acid used is appropriately in a range of, for example, 0.1 mol equivalent to 1000 mol equivalents based on 1 mol of Compound (II), preferably in a range of 1 mol equivalent to 100 mol equivalents based on 1 mol of Compound (II).

An organic amine can be used in combination with the acid described above. The organic amine is not particularly limited and includes, for example, triethylamine. The amount of the organic amine used is appropriately in a range of, e.g., 0.01 mol equivalent to 10 mol equivalents, and preferably in a range of 0.1 mol equivalent to 2 mol equivalents, based on 1 mol of the acid.

When a salt or mixture of the acid and the organic amine is used in this step, the salt or mixture includes, for example, a salt or mixture of trifluoroacetic acid and triethylamine, and more specifically, a mixture of 1 equivalent of triethylamine and 2 equivalents of trifluoroacetic acid.

The acid which can be used in this step may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, or a mixture thereof.

The reaction temperature in the reaction described above is preferably in a range of, e.g., 10° C. to 50° C., more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C.

The reaction time may vary depending upon kind of the acid used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

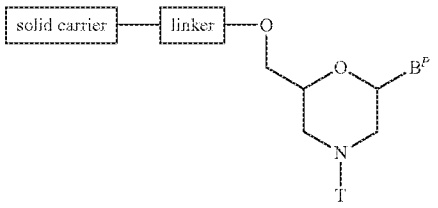
After completion of this step, a base may be added, if necessary, to neutralize the acid remained in the system. The "base" is not particularly limited and includes, for example, diisopropylamine. The base may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% (v/v) to 30% (v/v).

The solvent used in this step is not particularly limited so long as it is inert to the reaction, and includes dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, and a mixture thereof. The reaction temperature is preferably in a range of, e.g., 10° C. to 50° C., more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C.

The reaction time may vary depending upon kind of the base used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

In Compound (II), the compound of general formula (IIa) below (hereinafter Compound (IIa)), wherein n is 1 and L is a group (IV), can be produced by the following procedure.

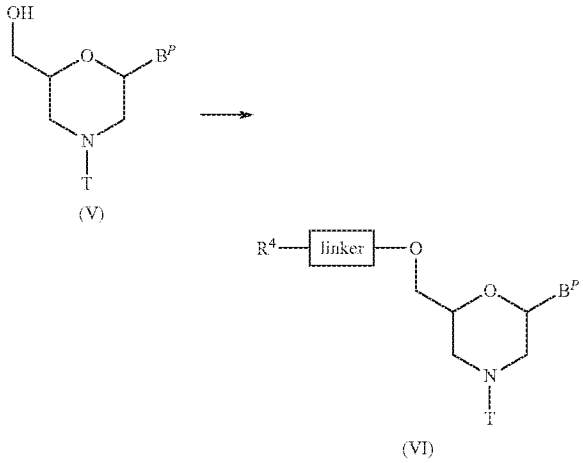
(IIa)



wherein B^P, T, linker and solid carrier have the same significance as defined above.

Step 1:

The compound represented by general formula (V) below is reacted with an acylating agent to prepare the compound represented by general formula (VI) below (hereinafter referred to as Compound (VI)).

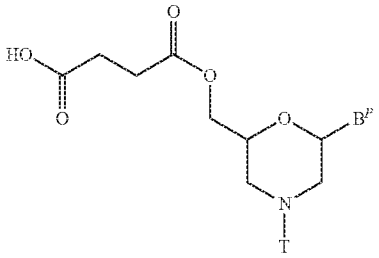


wherein B^P, T and linker have the same significance as defined above; and, R^4 represents hydroxy, a halogen or amino.

This step can be carried out by known procedures for introducing linkers, using Compound (V) as the starting material.

In particular, the compound represented by general formula (VIa) below can be produced by performing the method known as esterification, using Compound (V) and succinic anhydride.

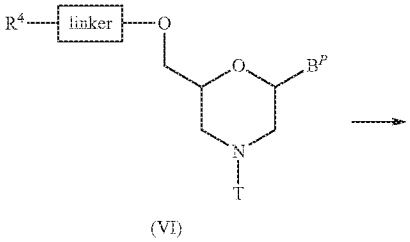
(VIa)



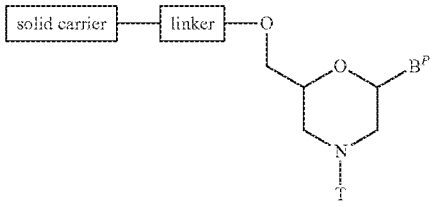
wherein B^P and T have the same significance as defined above.

Step 2:

Compound (VI) is reacted with a solid career by a condensing agent to prepare Compound (IIa).



(VI)



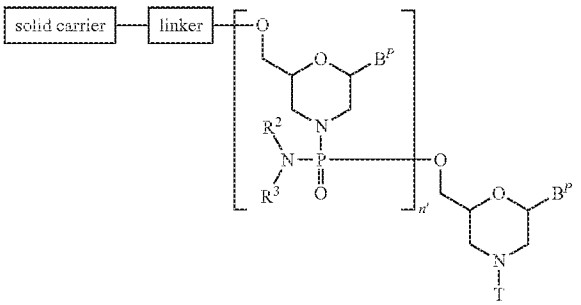
(IIa)

wherein B^P, R^4, T, linker and solid carrier have the same significance as defined above.

This step can be performed using Compound (VI) and a solid carrier in accordance with a process known as condensation reaction.

In Compound (II), the compound represented by general formula (IIa2) below wherein n is 2 to 99 and L is a group represented by general formula (IV) can be produced by using Compound (IIa) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

(IIa2)

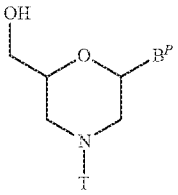


wherein B^P, R^2, R^3, T, linker and solid carrier have the same significance as defined above; and,

n' represents 1 to 98.

In Compound (II), the compound of general formula (IIb) below wherein n is 1 and L is hydrogen can be produced by the procedure described in, e.g., WO 1991/009033.

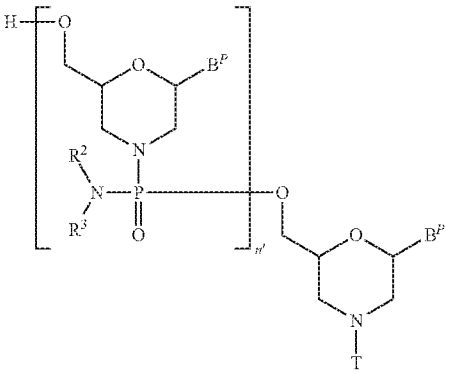
(IIb)



wherein B^P and T have the same significance as defined above.

In Compound (II), the compound represented by general formula (IIb2) below wherein n is 2 to 99 and L is hydrogen can be produced by using Compound (IIb) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

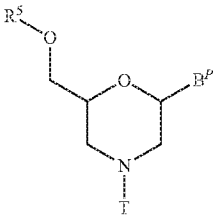
(IIb2)



wherein B^P, n', R^2, R^3 and T have the same significance as defined above.

In Compound (II), the compound represented by general formula (IIc) below wherein n is 1 and L is an acyl can be produced by performing the procedure known as acylation reaction, using Compound (IIb).

(IIc)

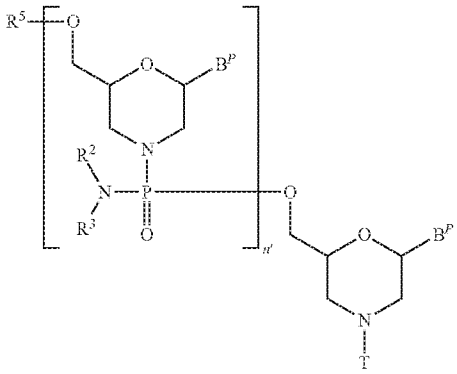


wherein B^P and T have the same significance as defined above; and,

R^5 represents an acyl.

In Compound (II), the compound represented by general formula (IIc2) below wherein n is 2 to 99 and L is an acyl can be produced by using Compound (IIc) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

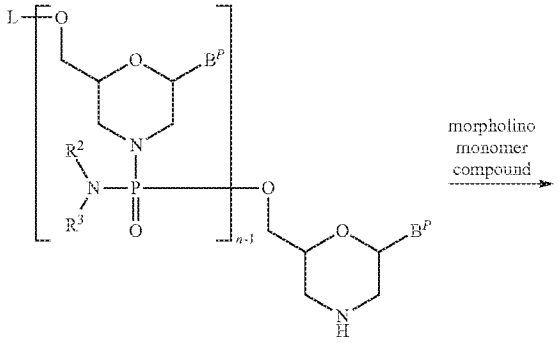
(IIc2)



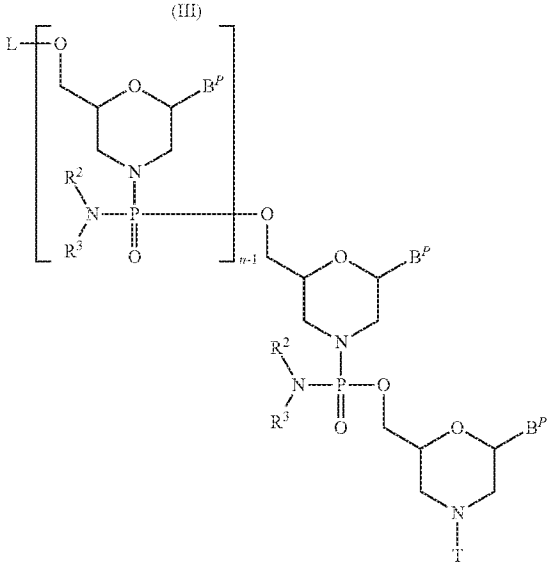
wherein B^P, n', R^2, R^3, R^5 and T have the same significance as defined above.

(2) Step B

Compound (III) is reacted with a morpholino monomer compound in the presence of a base to prepare the compound represented by general formula (VII) below (hereinafter referred to as Compound (VII)):



(III)

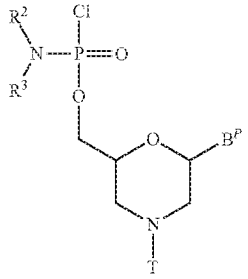


(VII)

wherein B^P, L, n, R^2, R^3 and T have the same significance as defined above.

This step can be performed by reacting Compound (III) with the morpholino monomer compound in the presence of a base.

The morpholino monomer compound includes, for example, compounds represented by general formula (VIII) below:



(VIII)

wherein B^P, R^2, R^3 and T have the same significance as defined above.

The "base" which can be used in this step includes, for example, diisopropylamine, triethylamine and N-ethylmorpholine. The amount of the base used is appropriately in a range of 1 mol equivalent to 1000 mol equivalents based on 1 mol of Compound (III), preferably, 10 mol equivalents to 100 mol equivalents based on 1 mol of Compound (III).

The morpholino monomer compound and base which can be used in this step may also be used as a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, N,N-dimethylimidazolidone, N-methylpiperidone, DMF, dichloromethane, acetonitrile, tetrahydrofuran, or a mixture thereof.

The reaction temperature is preferably in a range of e.g., 0° C. to 100° C., and more preferably, in a range of 10° C. to 50° C.

The reaction time may vary depending upon kind of the base used and reaction temperature, and is appropriately in a range of 1 minute to 48 hours in general, and preferably in a range of 30 minutes to 24 hours.

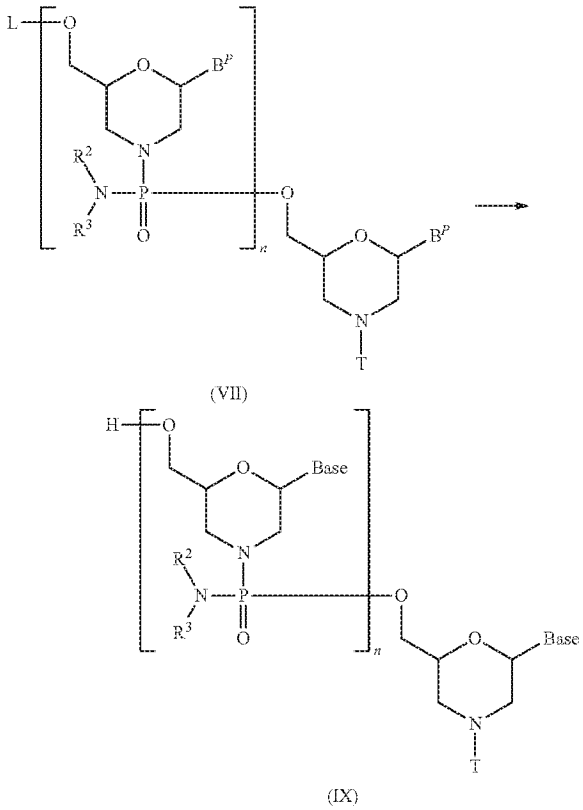
Furthermore, after completion of this step, an acylating agent can be added, if necessary. The "acylating agent" includes, for example, acetic anhydride, acetyl chloride and phenoxyacetic anhydride. The acylating agent may also be used as a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, dichloromethane, acetonitrile, an alcohol(s) (ethanol, isopropanol, trifluoroethanol, etc.), water, or a mixture thereof.

If necessary, a base such as pyridine, lutidine, collidine, triethylamine, diisopropylethylamine, N-ethylmorpholine, etc. may also be used in combination with the acylating agent. The amount of the acylating agent is appropriately in a range of 0.1 mol equivalent to 10000 mol equivalents, and preferably in a range of 1 mol equivalent to 1000 mol equivalents. The amount of the base is appropriately in a range of, e.g., 0.1 mol equivalent to 100 mol equivalents, and preferably in a range of 1 mol equivalent to 10 mol equivalents, based on 1 mol of the acylating agent.

The reaction temperature in this reaction is preferably in a range of 10° C. to 50° C., more preferably, in a range of 10° C. to 50° C., much more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C. The reaction time may vary depending upon kind of the acylating agent used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

(3) Step C:

In Compound (VII) produced in Step B, the protective group is removed using a deprotecting agent to prepare the compound represented by general formula (IX).



(VII)

(IX)

wherein Base, B^P, L, n, R^2, R^3 and T have the same significance as defined above.

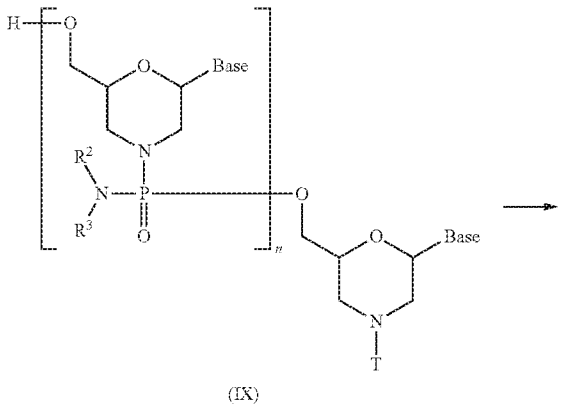
This step can be performed by reacting Compound (VII) with a deprotecting agent.

The "deprotecting agent" includes, e.g., conc. ammonia water and methylamine. The "deprotecting agent" used in this step may also be used as a dilution with, e.g., water, methanol, ethanol, isopropyl alcohol, acetonitrile, tetrahydrofuran, DMF, N,N-dimethylimidazolidone, N-methylpiperidone, or a mixture of these solvents. Among others, ethanol is preferred. The amount of the deprotecting agent used is appropriately in a range of, e.g., 1 mol equivalent to 100000 mol equivalents, and preferably in a range of 10 mol equivalents to 1000 mol equivalents, based on 1 mol of Compound (VII).

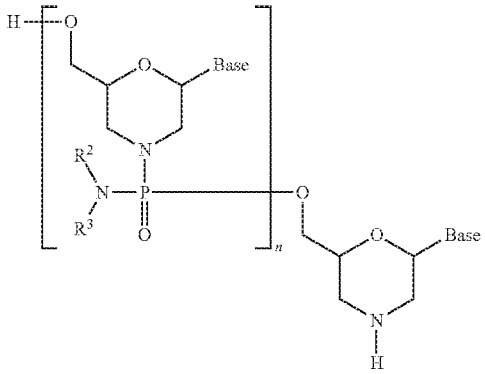
The reaction temperature is appropriately in a range of 15° C. to 75° C., preferably, in a range of 40° C. to 70° C., and more preferably, in a range of 50° C. to 60° C. The reaction time for deprotection may vary depending upon kind of Compound (VII), reaction temperature, etc., and is appropriately in a range of 10 minutes to 30 hours, preferably 30 minutes to 24 hours, and more preferably in a range of 5 hours to 20 hours.

(4) Step D:

PMO (I) is produced by reacting Compound (IX) produced in step C with an acid:



(IX)



(I)

wherein Base, n, R^2, R^3 and T have the same significance as defined above.

This step can be performed by adding an acid to Compound (IX).

The "acid" which can be used in this step includes, for example, trichloroacetic acid, dichloroacetic acid, acetic acid, phosphoric acid, hydrochloric acid, etc. The acid used is appropriately used to allow the solution to have a pH range of 0.1 to 4.0, and more preferably, in a range of pH 1.0 to 3.0. The solvent is not particularly limited so long as it is inert to the reaction, and includes, for example, acetonitrile, water, or a mixture of these solvents thereof.

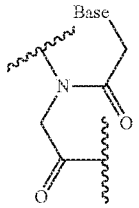
The reaction temperature is appropriately in a range of 10° C. to 50° C., preferably, in a range of 20° C. to 40° C., and more preferably, in a range of 25° C. to 35° C. The reaction time for deprotection may vary depending upon kind of Compound (IX), reaction temperature, etc., and is appropriately in a range of 0.1 minute to 5 hours, preferably 1 minute to 1 hour, and more preferably in a range of 1 minute to 30 minutes.

PMO (I) can be obtained by subjecting the reaction mixture obtained in this step to conventional means of separation and purification such as extraction, concentration, neutralization, filtration, centrifugal separation, recrystallization, reversed phase column chromatography C_8 to C_{18}, cation exchange column chromatography, anion exchange column chromatography, gel filtration column chromatography, high performance liquid chromatography, dialysis, ultrafiltration, etc., alone or in combination thereof. Thus, the desired PMO (I) can be isolated and purified (cf., e.g., WO 1991/09033).

In purification of PMO (I) using reversed phase chromatography, e.g., a solution mixture of 20 mM triethylamine/acetate buffer and acetonitrile can be used as an elution solvent.

In purification of PMO (I) using ion exchange chromatography, e.g., a solution mixture of 1 M saline solution and 10 mM sodium hydroxide aqueous solution can be used as an elution solvent.

A peptide nucleic acid is the oligomer of the present invention having a group represented by the following general formula as the constituent unit:



wherein Base has the same significance as defined above.

Peptide nucleic acids can be prepared by referring to, e.g., the following literatures.

1) P. E. Nielsen, M. Egholm, R. H. Berg, O. Buchardt, Science, 254, 1497 (1991)

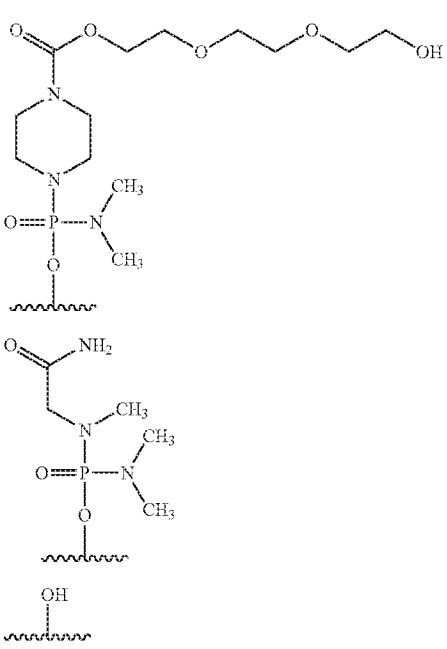
2) M. Egholm, O. Buchardt, P. E. Nielsen, R. H. Berg, Jacs., 114, 1895 (1992)

3) K. L. Dueholm, M. Egholm, C. Behrens, L. Christensen, H. F. Hansen, T. Vulpius, K. H. Petersen, R. H. Berg, P. E. Nielsen, O. Buchardt, J. Org. Chem., 59, 5767 (1994)

4) L. Christensen, R. Fitzpatrick, B. Gildea, K. H. Petersen, H. F. Hansen, T. Koch, M. Egholm, O. Buchardt, P. E. Nielsen, J. Coull, R. H. Berg, J. Pept. Sci., 1, 175 (1995)

5) T. Koch, H. F. Hansen, P. Andersen, T. Larsen, H. G. Batz, K. Otteson, H. Orum, J. Pept. Res., 49, 80 (1997)

In the oligomer of the present invention, the 5' end may be any of chemical structures (1) to (3) below, and preferably is (3)-OH.



(1)

(2)

(3)

Hereinafter, the groups shown by (1), (2) and (3) above are referred to as "Group (1)," "Group (2)" and "Group (3)," respectively.

2. Pharmaceutical Composition

The oligomer of the present invention causes exon 53 skipping with a higher efficiency as compared to the prior art antisense oligomers. It is thus expected that conditions of muscular dystrophy can be relieved with high efficience by administering the pharmaceutical composition comprising the oligomer of the present invention to DMD patients. For example, when the pharmaceutical composition comprising the oligomer of the present invention is used, the same therapeutic effects can be achieved even in a smaller dose than that of the oligomers of the prior art. Accordingly, side effects can be alleviated and such is economical.

In another embodiment, the present invention provides the pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the oligomer of the present invention, a pharmaceutically acceptable salt or hydrate thereof (hereinafter referred to as "the composition of the present invention").

Examples of the pharmaceutically acceptable salt of the oligomer of the present invention contained in the composition of the present invention are alkali metal salts such as salts of sodium, potassium and lithium; alkaline earth metal salts such as salts of calcium and magnesium; metal salts such as salts of aluminum, iron, zinc, copper, nickel, cobalt, etc.; ammonium salts; organic amine salts such as salts of t-octylamine, dibenzylamine, morpholine, glucosamine, phenylglycine alkyl ester, ethylenediamine, N-methylglucamine, guanidine, diethylamine, triethylamine, dicyclohexylamine, N,N'-dibenzylethylenediamine, chloroprocaine, procaine, diethanolamine, N-benzylphenethylamine, piperazine, tetramethylammonium, tris(hydroxymethyl)aminomethane; hydrohalide salts such as salts of hydrofluorates, hydrochlorides, hydrobromides and hydroiodides; inorganic acid salts such as nitrates, perchlorates, sulfates, phosphates, etc.; lower alkane sulfonates such as methanesulfonates, trifluoromethanesulfonates and ethanesulfonates; arylsulfonates such as benzenesulfonates and p-toluenesulfonates; organic acid salts such as acetates, malates, fumarates, succinates, citrates, tartarates, oxalates, maleates, etc.; and, amino acid salts such as salts of glycine, lysine, arginine, ornithine, glutamic acid and aspartic acid. These salts may be produced by known methods. Alternatively, the oligomer of the present invention contained in the composition of the present invention may be in the form of a hydrate thereof.

Administration route for the composition of the present invention is not particularly limited so long as it is pharmaceutically acceptable route for administration, and can be chosen depending upon method of treatment. In view of easiness in delivery to muscle tissues, preferred are intravenous administration, intraarterial administration, intramuscular administration, subcutaneous administration, oral administration, tissue administration, transdermal administration, etc. Also, dosage forms which are available for the composition of the present invention are not particularly limited, and include, for example, various injections, oral agents, drips, inhalations, ointments, lotions, etc.

In administration of the oligomer of the present invention to patients with muscular dystrophy, the composition of the present invention preferably contains a carrier to promote delivery of the oligomer to muscle tissues. Such a carrier is not particularly limited as far as it is pharmaceutically acceptable, and examples include cationic carriers such as cationic liposomes, cationic polymers, etc., or carriers using viral envelope. The cationic liposomes are, for example, liposomes composed of 2-O-(2-diethylaminoethyl)carabamoyl-1,3-O-dioleoylglycerol and phospholipids as the essential constituents (hereinafter referred to as "liposome A"), Oligofectamine (registered trademark) (manufactured by Invitrogen Corp.), Lipofectin (registered trademark) (manufactured by Invitrogen Corp.), Lipofectamine (registered trademark) (manufactured by Invitrogen Corp.), Lipofectamine 2000 (registered trademark) (manufactured by Invitrogen Corp.), DMRIE-C (registered trademark) (manufactured by Invitrogen Corp.), GeneSilencer (registered trademark) (manufactured by Gene Therapy Systems), TransMessenger (registered trademark) (manufactured by QIAGEN, Inc.), TransIT TKO (registered trademark) (manufactured by Minis) and Nucleofector II (Lonza). Among others, liposome A is preferred. Examples of cationic polymers are JetSI (registered trademark) (manufactured by Qbiogene, Inc.) and Jet-PEI (registered trademark) (polyethylenimine, manufactured by Qbiogene, Inc.). An example of carriers using viral envelop is GenomeOne (registered trademark) (HVJ-E liposome, manufactured by Ishihara Sangyo). Alternatively, the medical devices described in Japanese Patent No. 2924179 and the cationic carriers described in Japanese Domestic Re-Publication PCT Nos. 2006/129594 and 2008/096690 may be used as well.

A concentration of the oligomer of the present invention contained in the composition of the present invention may vary depending on kind of the carrier, etc., and is appropriately in a range of 0.1 nM to 100 μM, preferably in a range of 1 nM to 10 μM, and more preferably in a range of 10 nM to 1 μM. A weight ratio of the oligomer of the present invention contained in the composition of the present invention and the carrier (carrier/oligomer of the present invention) may vary depending on property of the oligomer, type of the carrier, etc., and is appropriately in a range of 0.1 to 100, preferably in a range of 1 to 50, and more preferably in a range of 10 to 20.

In addition to the oligomer of the present invention and the carrier described above, pharmaceutically acceptable additives may also be optionally formulated in the composition of the present invention. Examples of such additives are emulsification aids (e.g., fatty acids having 6 to 22 carbon atoms and their pharmaceutically acceptable salts, albumin and dextran), stabilizers (e.g., cholesterol and phosphatidic acid), isotonizing agents (e.g., sodium chloride, glucose, maltose, lactose, sucrose, trehalose), and pH controlling agents (e.g., hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid, sodium hydroxide, potassium hydroxide and triethanolamine). One or more of these additives can be used. The content of the additive in the composition of the present invention is appropriately 90 wt % or less, preferably 70 wt % or less and more preferably, 50 wt % or less.

The composition of the present invention can be prepared by adding the oligomer of the present invention to a carrier dispersion and adequately stirring the mixture. Additives may be added at an appropriate step either before or after addition of the oligomer of the present invention. An aqueous solvent that can be used in adding the oligomer of the present invention is not particularly limited as far as it is pharmaceutically acceptable, and examples are injectable water or injectable distilled water, electrolyte fluid such as physiological saline, etc., and sugar fluid such as glucose fluid, maltose fluid, etc. A person skilled in the art can appropriately choose conditions for pH and temperature for such matter.

The composition of the present invention may be prepared into, e.g., a liquid form and its lyophilized preparation. The lyophilized preparation can be prepared by lyophilizing the composition of the present invention in a liquid form in a conventional manner. The lyophilization can be performed, for example, by appropriately sterilizing the composition of the present invention in a liquid form, dispensing an aliquot

into a vial container, performing preliminary freezing for 2 hours at conditions of about −40 to −20° C., performing a primary drying at 0 to 10° C. under reduced pressure, and then performing a secondary drying at about 15 to 25° C. under reduced pressure. In general, the lyophilized preparation of the composition of the present invention can be obtained by replacing the content of the vial with nitrogen gas and capping.

The lyophilized preparation of the composition of the present invention can be used in general upon reconstitution by adding an optional suitable solution (reconstitution liquid) and redissolving the preparation. Such a reconstitution liquid includes injectable water, physiological saline and other infusion fluids. A volume of the reconstitution liquid may vary depending on the intended use, etc., is not particularly limited, and is suitably 0.5 to 2-fold greater than the volume prior to lyophilization or no more than 500 mL.

It is desired to control a dose of the composition of the present invention to be administered, by taking the following factors into account: the type and dosage form of the oligomer of the present invention contained; patients' conditions including age, body weight, etc.; administration route; and the characteristics and extent of the disease. A daily dose calculated as the amount of the oligomer of the present invention is generally in a range of 0.1 mg to 10 g/human, and preferably 1 mg to 1 g/human. This numerical range may vary occasionally depending on type of the target disease, administration route and target molecule. Therefore, a dose lower than the range may be sufficient in some occasion and conversely, a dose higher than the range may be required occasionally. The composition can be administered from once to several times daily or at intervals from one day to several days.

In still another embodiment of the composition of the present invention, there is provided a pharmaceutical composition comprising a vector capable of expressing the oligonucleotide of the present invention and the carrier described above. Such an expression vector may be a vector capable of expressing a plurality of the oligonucleotides of the present invention. The composition may be formulated with pharmaceutically acceptable additives as in the case with the composition of the present invention containing the oligomer of the present invention. A concentration of the expression vector contained in the composition may vary depending upon type of the career, etc., and is appropriately in a range of 0.1 nM to 100 μM, preferably in a range of 1 nM to 10 μM, and more preferably in a range of 10 nM to 1 μM. A weight ratio of the expression vector contained in the composition and the carrier (carrier/expression vector) may vary depending on property of the expression vector, type of the carrier, etc., and is appropriately in a range of 0.1 to 100, preferably in a range of 1 to 50, and more preferably in a range of 10 to 20. The content of the carrier contained in the composition is the same as in the case with the composition of the present invention containing the oligomer of the present invention, and a method for producing the same is also the same as in the case with the composition of the present invention.

Hereinafter, the present invention will be described in more detail with reference to EXAMPLES and TEST EXAMPLES below, but is not deemed to be limited thereto.

EXAMPLES

Reference Example 1

4-{[(2S,6R)-6-(4-Benzamido-2-oxopyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin

Step 1: Production of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid

Under argon atmosphere, 22.0 g of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide and 7.04 g of 4-dimethylaminopyridine (4-DMAP) were suspended in 269 mL of dichloromethane, and 5.76 g of succinic anhydride was added to the suspension, followed by stirring at room temperature for 3 hours. To the reaction solution was added 40 mL of methanol, and the mixture was concentrated under reduced pressure. The residue was extracted using ethyl acetate and 0.5M aqueous potassium dihydrogenphosphate solution. The resulting organic layer was washed sequentially with 0.5M aqueous potassium dihydrogenphosphate solution, water and brine in the order mentioned. The resulting organic layer was dried over sodium sulfate and concentrated under reduced pressure to give 25.9 g of the product.

Step 2: Production of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin

After 23.5 g of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid was dissolved in 336 mL of pyridine (dehydrated), 4.28 g of 4-DMAP and 40.3 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were added to the solution. Then, 25.0 g of Aminomethyl Polystyrene Resin cross-linked with 1% DVB (manufactured by Tokyo Chemical Industry Co., Ltd., A1543) and 24 mL of triethylamine were added to the mixture, followed by shaking at room temperature for 4 days. After completion of the reaction, the resin was taken out by filtration. The resulting resin was washed sequentially with pyridine, methanol and dichloromethane in the order mentioned, and dried under reduced pressure. To the resulting resin were added 150 mL of tetrahydrofuran (dehydrate), 15 mL of acetic anhydride and 15 mL of 2,6-lutidine, and the mixture was shaken at room temperature for 2 hours. The resin was taken out by filtration, washed sequentially with pyridine, methanol and dichloromethane in the order mentioned, and dried under reduced pressure to give 33.7 g of the product.

The loading amount of the product was determined by measuring UV absorbance at 409 nm of the molar amount of the trityl per g resin using a known method. The loading amount of the resin was 397.4 μmol/g.

Conditions of UV measurement

Device: U-2910 (Hitachi, Ltd.)

Solvent: methanesulfonic acid

Wavelength: 265 nm

ε Value: 45000

Reference Example 2

4-Oxo-4-{[(2S,6R)-6-(6-oxo-2-[2-phenoxyacetamido]-1H-purin-9-yl)-4-tritylmorpholin-2-yl]methoxy}butanoic acid loaded onto 2-aminomethylpolystyrene resin

Step 1: Production of N^2-(phenoxyacetyl)guanosine

Guanosine, 100 g, was dried at 80° C. under reduced pressure for 24 hours. After 500 mL of pyridine (anhydrous) and 500 mL of dichloromethane (anhydrous) were added thereto, 401 mL of chlorotrimethylsilane was dropwise added to the mixture under an argon atmosphere at 0° C., followed by stirring at room temperature for 3 hours. The mixture was again ice-cooled and 66.3 g of phenoxyacetyl chloride was dropwise added thereto. Under ice cooling, the mixture was stirred for further 3 hours. To the reaction solution was added 500 mL of methanol, and the mixture was stirred at room temperature overnight. The solvent was then removed by distillation under reduced pressure. To the residue was added 500 mL of methanol, and concentration under reduced pressure was performed 3 times. To the residue was added 4 L of water, and the mixture was stirred for an hour under ice cooling. The precipitates formed were taken out by filtration, washed sequentially with water and cold methanol and then dried to give 150.2 g of the objective compound (yield: 102%) (cf.: Org. Lett. (2004), Vol. 6, No. 15, 2555-2557).

Step 2

N-{9-[(2R,6S)-6-(hydroxymethyl)-4-morpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide p-toluenesulfonate

In 480 mL of methanol was suspended 30 g of the compound obtained in Step 1, and 130 mL of 2N hydrochloric acid was added to the suspension under ice cooling. Subsequently, 56.8 g of ammonium tetraborate tetrahydrate and 16.2 g of sodium periodate were added to the mixture in the order mentioned and stirred at room temperature for 3 hours. The reaction solution was ice cooled and the insoluble matters were removed by filtration, followed by washing with 100 mL of methanol. The filtrate and washing liquid were combined and the mixture was ice cooled. To the mixture was added 11.52 g of 2-picoline borane. After stirring for 20 minutes, 54.6 g of p-toluenesulfonic acid monohydrate was slowly added to the mixture, followed by stirring at 4° C. overnight. The precipitates were taken out by filtration and washed with 500 mL of cold methanol and dried to give 17.7 g of the objective compound (yield: 43.3%).

^{1}H NMR (6, DMSO-d6): 9.9-9.2 (2H, br), 8.35 (1H, s), 7.55 (2H, m), 7.35 (2H, m), 7.10 (2H, d, J=7.82 Hz), 7.00 (3H, m), 5.95 (1H, dd, J=10.64, 2.42 Hz), 4.85 (2H, s), 4.00 (1H, m), 3.90-3.60 (2H, m), 3.50-3.20 (5H, m), 2.90 (1H, m), 2.25 (3H, s)

Step 3: Production of N-{9-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide

In 30 mL of dichloromethane was suspended 2.0 g of the compound obtained in Step 2, and 13.9 g of triethylamine and 18.3 g of trityl chloride were added to the suspension under ice cooling. The mixture was stirred at room temperature for an hour. The reaction solution was washed with saturated sodium bicarbonate aqueous solution and then with water, and dried. The organic layer was concentrated under reduced pressure. To the residue was added 40 mL of 0.2M sodium citrate buffer (pH 3)/methanol (1:4 (v/v)), and the mixture was stirred. Subsequently, 40 mL of water was added and the mixture was stirred for an hour under ice cooling. The mixture was taken out by filtration, washed with cold methanol and dried to give 1.84 g of the objective compound (yield: 82.0%).

Step 4: Production of 4-oxo-4-{[(2S,6R)-6-(6-oxo-2-[2-phenoxyacetamido]-1H-purin-9-yl)-4-tritylmorpholin-2-yl]methoxy}butanoic acid loaded onto aminomethyl polystyrene resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that N-{9-[2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of REFERENCE EXAMPLE 1.

Reference Example 3

4-{[(2S,6R)-6-(5-Methyl-2,4-dioxo-3,4-dihydropyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that 1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-5-methylpyrimidine-2,4(1H,3H)-dione was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of REFERENCE EXAMPLE 1.

Reference Example 4

1,12-Dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that 2-[2-(2-hydroxyethoxy)ethoxy]ethyl 4-tritylpiperazine-1-carboxylic acid (the compound described in WO 2009/064471) was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide.

According to the descriptions in EXAMPLES 1 to 12 and REFERENCE EXAMPLES 1 to 3 below, various types of PMO shown by PMO Nos. 1-11 and 13-16 in TABLE 2 were synthesized. The PMO synthesized was dissolved in injectable water (manufactured by Otsuka Pharmaceutical Factory, Inc.). PMO No. 12 was purchased from Gene Tools, LLC.

TABLE 2

PMO No.	Target sequence in exon 53	Note	SEQ ID NO:
1	31-55	5' end: group (3)	SEQ ID NO: 4
2	32-53	5' end: group (3)	SEQ ID NO: 8
3	32-56	5' end: group (3)	SEQ ID NO: 11
4	33-54	5' end: group (3)	SEQ ID NO: 15
5	34-58	5' end: group (3)	SEQ ID NO: 25
6	36-53	5' end: group (3)	SEQ ID NO: 32
7	36-55	5' end: group (3)	SEQ ID NO: 34
8	36-56	5' end: group (3)	SEQ ID NO: 35
9	36-57	5' end: group (3)	SEQ ID NO: 36

TABLE 2-continued

PMO No.	Target sequence in exon 53	Note	SEQ ID NO:
10	33-57	5' end: group (3)	SEQ ID NO: 18
11	39-69	Sequence corresponding to H53A(+39 + 69) (cf. Table 1) in Non-Patent Document 3, 5' end: group (3)	SEQ ID NO: 38
12	30-59	Sequence corresponding to h53A30/1 (cf. Table 1) in Non-Patent Document 5, 5' end: group (2)	SEQ ID NO: 39
13	32-56	5' end: group (1)	SEQ ID NO: 11
14	36-56	5' end: group (1)	SEQ ID NO: 35
15	30-59	Sequence corresponding to h53A30/1 (cf. Table 1) in Non-Patent Document 5 5' end: group (3)	SEQ ID NO: 39
16	23-47	Sequence corresponding to SEQ ID NO: 429 described in Patent Document 4, 5' end: group (3)	SEQ ID NO: 47

Example 1

PMO No. 8

4-{[(2S,6R)-6-(4-Benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid, loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 1), 2 g (800 μmol) was transferred to a reaction vessel, and 30 mL of dichloromethane was added thereto. The mixture was allowed to stand for 30 minutes. After the mixture was further washed twice with 30 mL of dichloromethane, the following synthesis cycle was started. The desired morpholino monomer compound was added in each cycle to give the nucleotide sequence of the title compound.

TABLE 3

Step	Reagent	Volume (mL)	Time (min)
1	deblocking solution	30	2.0
2	deblocking solution	30	2.0
3	deblocking solution	30	2.0
4	deblocking solution	30	2.0
5	deblocking solution	30	2.0
6	deblocking solution	30	2.0
7	neutralizing solution	30	1.5
8	neutralizing solution	30	1.5
9	neutralizing solution	30	1.5
10	neutralizing solution	30	1.5
11	neutralizing solution	30	1.5
12	neutralizing solution	30	1.5
13	dichloromethane	30	0.5
14	dichloromethane	30	0.5
15	dichloromethane	30	0.5
16	coupling solution B	20	0.5
17	coupling solution A	6-11	90.0
18	dichloromethane	30	0.5
19	dichloromethane	30	0.5
20	dichloromethane	30	0.5
21	capping solution	30	3.0
22	capping solution	30	3.0
23	dichloromethane	30	0.5
24	dichloromethane	30	0.5
25	dichloromethane	30	0.5

The deblocking solution used was a solution obtained by dissolving a mixture of trifluoroacetic acid (2 equivalents) and triethylamine (1 equivalent) in a dichloromethane solution containing 1% (v/v) ethanol and 10% (v/v) 2,2,2-trifluoroethanol to be 3% (w/v). The neutralizing solution used was a solution obtained by dissolving N,N-diisopropylethylamine in a dichloromethane solution containing 25% (v/v) 2-propanol to be 5% (v/v). The coupling solution A used was a solution obtained by dissolving the morpholino monomer compound in 1,3-dimethyl-2-imidazolidinone containing 10% (v/v) N,N-diisopropylethylamine to be 0.15M. The coupling solution B used was a solution obtained by dissolving N,N-diisopropylethylamine in 1,3-dimethyl-2-imidazolidinone to be 10% (v/v). The capping solution used was a solution obtained by dissolving 20% (v/v) acetic anhydride and 30% (v/v) 2,6-lutidine in dichloromethane.

The aminomethyl polystyrene resin loaded with the PMO synthesized above was recovered from the reaction vessel and dried at room temperature for at least 2 hours under reduced pressure. The dried PMO loaded onto aminomethyl polystyrene resin was charged in a reaction vessel, and 200 mL of 28% ammonia water-ethanol (1/4) was added thereto. The mixture was stirred at 55° C. for 15 hours. The aminomethyl polystyrene resin was separated by filtration and washed with 50 mL of water-ethanol (1/4). The resulting filtrate was concentrated under reduced pressure. The resulting residue was dissolved in 100 mL of a solvent mixture of 20 mM acetic acid-triethylamine buffer (TEAA buffer) and acetonitrile (4/1) and filtered through a membrane filter. The filtrate obtained was purified by reversed phase HPLC. The conditions used are as follows.

TABLE 4

Column	XTerra MS18 (Waters, ϕ50x 100 mm, 1CV = 200 mL)
Flow rate	60 mL/min
Column temperature	room temperature
Solution A	20 mM TEAA buffer
Solution B	CH_3CN
Gradient	(B) conc. 20→50%/9CV

Each fraction was analyzed and the product was recovered in 100 mL of acetonitrile-water (1/1), to which 200 mL of ethanol was added. The mixture was concentrated under reduced pressure. Further drying under reduced pressure gave a white solid. To the resulting solid was added 300 mL of 10 mM phosphoric acid aqueous solution to suspend the solid. To the suspension was added 10 mL of 2M phosphoric acid aqueous solution, and the mixture was stirred for 15 minutes. Furthermore, 15 mL of 2M sodium hydrate aqueous solution was added for neutralization. Then, 15 mL of 2M sodium hydroxide aqueous solution was added to make the mixture alkaline, followed by filtration through a membrane filter (0.45 μm). The mixture was thoroughly washed with 100 mL of 10 mM sodium hydroxide aqueous solution to give the product as an aqueous solution.

The resulting aqueous solution containing the product was purified by an anionic exchange resin column. The conditions used are as follows.

TABLE 5

Column	Source 30Q (GE Healthcare, ϕ40x 150 mm, 1CV = 200 mL)
Flow rate	80 mL/min
Column temp.	room temperature
Solution A	10 mM sodium hydroxide aqueous solution
Solution B	10 mM sodium hydroxide aqueous solution, 1M sodium chloride aqueous solution
Gradient	(B) conc. 5→35%/15CV

Each fraction was analyzed (on HPLC) and the product was obtained as an aqueous solution. To the resulting aqueous solution was added 225 mL of 0.1M phosphate buffer (pH 6.0) for neutralization. The mixture was filtered through a membrane filter (0.45 μm). Next, ultrafiltration was performed under the conditions described below.

TABLE 6

Filter	PELLICON2 MINI FILTER PLBC 3K Regenerated Cellulose, Screen Type C
Size	0.1 m^2

The filtrate was concentrated to give approximately 250 mL of an aqueous solution. The resulting aqueous solution was filtered through a membrane filter (0.45 μm). The aqueous solution obtained was freeze-dried to give 1.5 g of the objective compound as a white cotton-like solid.

ESI-TOF-MS Calcd.: 6924.82.

Found: 6923.54.

Example 2

PMO. No. 1

The title compound was produced in accordance with the procedure of EXAMPLE 1.

MALDI-TOF-MS Calcd.: 8291.96.

Found: 8296.24.

Example 3

PMO. No. 2

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 7310.13.

Found: 7309.23.

Example 4

PMO. No. 3

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 8270.94.

Found: 8270.55.

Example 5

PMO. No. 4

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl)methoxy)-4-oxobutanoic acid (REFERENCE EXAMPLE 3) loaded onto aminomethyl polystyrene resin was used as the starting material.

ESI-TOF-MS Calcd.: 7310.13.

Found: 7310.17.

Example 6

PMO. No. 5

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl)methoxy)-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 3) was used as the starting material.

ESI-TOF-MS Calcd.: 8270.94.

Found: 8270.20.

Example 7

PMO. No. 6

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 5964.01.

Found: 5963.68.

Example 8

PMO. No. 7

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 6609.55.

Found: 6608.85.

Example 9

PMO. No. 9

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-oxo-4-(((2S,6R)-6-(6-oxo-2-(2-phenoxyacetamido)-1H-purin-9(6H)-yl)-4-tritylmorpholin-2-yl)methoxy)butanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 2) was used as the starting material.

ESI-TOF-MS Calcd.: 7280.11.

Found: 7279.42.

Example 10

PMO. No. 10

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-oxo-4-(((2S,6R)-6-(6-oxo-2-(2-phenoxyacetamido)-1H-purin-9(6H)-yl)-4-tritylmorpholin-2-yl)methoxy)butanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 2) was used as the starting material.

ESI-TOF-MS Calcd.: 8295.95.

Found: 8295.91.

Example 11

PMO. No. 13

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 1,12-dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 4) was used as the starting material.

ESI-TOF-MS Calcd.: 7276.15.

Found: 7276.69.

Example 12

PMO. No. 14

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 1,12-dioxo-1-(4-tri-

tylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 4) was used as the starting material.

ESI-TOF-MS Calcd.: 8622.27.

Found: 8622.29.

Comparative Example 1

PMO. No. 11

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 10274.63.

Found: 10273.71.

Comparative Example 2

PMO. No. 15

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 9941.33.

Found: 9940.77.

Comparative Example 3

PMO. No. 16

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 8238.94.

Found: 8238.69.

Test Example 1

In Vitro Assay

Using an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza), 10 μM of the oligomers PMO Nos. 1 to 8 of the present invention and the antisense oligomer PMO No. 11 were transfected with 4×10^5 of RD cells (human rhabdomyosarcoma cell line). The Program T-030 was used.

After transfection, the cells were cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen) under conditions of 37° C. and 5% CO_2. The cells were washed twice with PBS (manufactured by Nissui, hereinafter the same) and 500 μl of ISOGEN (manufactured by Nippon Gene) was added to the cells. After the cells were allowed to stand at room temperature for a few minutes to lyse the cells, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a Titan One Tube RT-PCR Kit (manufactured by Roche). A reaction solution was prepared in accordance with the protocol attached to the kit. A PTC-100 (manufactured by MJ Research) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

94° C., 2 mins: thermal denaturation

[94° C., 10 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

(SEQ ID NO: 40)
Forward primer: 5'-AGGATTTGGAACAGAGGCGTC-3'

(SEQ ID NO: 41)
Reverse primer: 5'-GTCTGCCACTGGCGGAGGTC-3'

Next, a nested PCR was performed with the product amplified by RT-PCR above using a Taq DNA Polymerase (manufactured by Roche). The PCR program used is as follows.

94° C., 2 mins: thermal denaturation

[94° C., 15 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above are given below.

(SEQ ID NO: 42)
Forward primer: 5'-CATCAAGCAGAAGGCAACAA-3'

(SEQ ID NO: 43)
Reverse primer: 5'-GAAGTTTCAGGGCCAAGTCA-3'

The reaction product, 1 of the nested PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)}=A/(A+B)\times100$$

Experimental Results

The results are shown in FIG. **1**. This experiment revealed that the oligomers PMO Nos. 1 to 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the antisense oligomer PMO No. 11. In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than four times higher exon skipping efficiency than that of the antisense oligomer PMO No. 11.

Test Example 2

In Vitro Assay Using Human Fibroblasts

Human myoD gene (SEQ ID NO: 44) was introduced into TIG-119 cells (human normal tissue-derived fibroblasts, National Institute of Biomedical Innovation) or 5017 cells (human DMD patient-derived fibroblasts, Coriell Institute for Medical Research) using a ZsGreen1 coexpression retroviral vector.

After incubation for 4 to 5 days, ZsGreen-positive MyoD-transformed fibroblasts were collected by FACS and plated at $5\times10^4/cm^2$ into a 12-well plate. As a growth medium, there was used 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen Corp.) containing 10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

The medium was replaced 24 hours later by differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). The medium was exchanged every 2 to 3 days and incubation was continued for 12 to 14 days to differentiate into myotubes.

Subsequently, the differentiation medium was replaced by a differentiation medium containing 6 μM Endo-Porter (Gene Tools), and the morpholino oligomer was added thereto in a final concentration of 10 μM. After incubation for 48 hours, total RNA was extracted from the cells using a TRIzol (manufactured by Invitrogen Corp.). RT-PCR was performed with 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. A reaction solution was prepared in accordance with the protocol attached to the kit. An iCycler (manufactured by Bio-Rad) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 1 mins; 60° C., 1 mins; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 7 mins: final extension

The primers used were hEX51F and hEX55R.

(SEQ ID NO: 45)
hEX51F: 5'-CGGGCTTGGACAGAACTTAC-3'

(SEQ ID NO: 46)
hEx55R: 5'-TCCTTACGGGTAGCATCCTG-3'

The reaction product of RT-PCR above was separated by 2% agarose gel electrophoresis and gel images were captured with a GeneFlash (Syngene). The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured using an Image J (manufactured by National Institutes of Health). Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation.

$$\text{Skipping efficiency (\%)}=A/(A+B)\times 100$$

Experimental Results

The results are shown in FIGS. **2** and **3**. This experiment revealed that in TIG-119 cells, the oligomers PMO Nos. 3, 8 and 9 of the present invention (FIG. **2**) all caused exon 53 skipping with a higher efficiency than the antisense oligomer PMO No. 12 (FIG. **2**). In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than twice higher exon skipping efficiency than that of the antisense oligomer PMO No. 12 (FIG. **2**).

Furthermore, this experiment revealed that the oligomers PMO Nos. 3 and 8 to 10 of the present invention (FIG. **3**) all caused exon 53 skipping with a higher efficiency than the antisense oligomer PMO No. 12 (FIG. **3**). In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than seven times higher exon skipping efficiency than that of the antisense oligomer PMO No. 12 (FIG. **3**).

Test Example 3

In Vitro Assay Using Human Fibroblasts

The skin fibroblast cell line (fibroblasts from human DMD patient (exons 45-52 or exons 48-52)) was established by biopsy from the medial left upper arm of DMD patient with deletion of exons 45-52 or DMD patient with deletion of exons 48-52. Human myoD gene (SEQ ID NO: 44) was introduced into the fibroblast cells using a ZsGreen1 coexpression retroviral vector.

After incubation for 4 to 5 days, ZsGreen-positive MyoD-transformed fibroblasts were collected by FACS and plated at $5\times10^4/cm^2$ into a 12-well plate. As a growth medium, there was used 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen Corp.) containing 10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

The medium was replaced 24 hours later by a differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). The medium was exchanged every 2 to 3 days and incubation was continued for 12, 14 or 20 days to differentiate into myotubes.

Subsequently, the differentiation medium was replaced by a differentiation medium containing 6 μM Endo-Porter (Gene Tools), and a morpholino oligomer was added thereto at a final concentration of 10 μM. After incubation for 48 hours, total RNA was extracted from the cells using a TRIzol (manufactured by Invitrogen Corp.). RT-PCR was performed with 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. A reaction solution was prepared in accordance with the protocol attached to the kit. An iCycler (manufactured by Bio-Rad) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 1 mins; 60° C., 1 mins; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 7 mins: final extension

The primers used were hEx44F and h55R.

(SEQ ID NO: 48)
hEx44F: 5'-TGTTGAGAAATGGCGGCGT-3'

(SEQ ID NO: 46)
hEx55R: 5'-TCCTTACGGGTAGCATCCTG-3'

The reaction product of RT-PCR above was separated by 2% agarose gel electrophoresis and gel images were captured with a GeneFlash (Syngene). The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured using an Image J (manufactured by National Institutes of Health). Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation.

$$\text{Skipping efficiency (\%)}=A/(A+B)\times 100$$

Experimental Results

The results are shown in FIGS. **4** and **5**. This experiment revealed that the oligomers PMO Nos. 3 and 8 of the present invention caused exon 53 skipping with an efficiency as high as more than 80% in the cells from DMD patient with deletion of exons 45-52 (FIG. **4**) or deletion of exons 48-52 (FIG. **5**). Also, the oligomers PMO Nos. 3 and 8 of the present invention were found to cause exon 53 skipping with a higher efficiency than that of the antisense oligomer PMO No. 15 in the cells from DMD patient with deletion of exons 45-52 (FIG. **4**).

Test Example 4

Western Blotting

The oligomer PMO No. 8 of the present invention was added to the cells at a concentration of 10 μM, and proteins were extracted from the cells after 72 hours using a RIPA buffer (manufactured by Thermo Fisher Scientific) containing Complete Mini (manufactured by Roche Applied Science) and quantified using a BCA protein assay kit (manufactured by Thermo Fisher Scientific). The proteins were

electrophoresed in NuPAGE Novex Tris-Acetate Gel 3-8% (manufactured by Invitrogen) at 150V for 75 minutes and transferred onto a PVDF membrane (manufactured by Millipore) using a semi-dry blotter. The PVDF membrane was blocked with a 5% ECL Blocking agent (manufactured by GE Healthcare) and the membrane was then incubated in a solution of anti-dystrophin antibody (manufactured by NCL-Dysl, Novocastra). After further incubation in a solution of peroxidase-conjugated goat-antimouse IgG (Model No. 170-6516, Bio-Rad), the membrane was stained with ECL Plus Western blotting system (manufactured by GE Healthcare).

Immunostaining

The oligomer PMO No. 3 or 8 of the present invention was added to the cells. The cells after 72 hours were fixed in 3% paraformaldehyde for 10 minutes, followed by incubation in 10% Triton-X for 10 minutes. After blocking in 10% goat serum-containing PBS, the membrane was incubated in a solution of anti-dystrophin antibody (NCL-Dysl, Novocastra). The membrane was further incubated in a solution of anti-mouse IgG antibody (manufactured by Invitrogen). The membrane was mounted with Pro Long Gold Antifade reagent (manufactured by Invitrogen) and observed with a fluorescence microscope.

Experimental Results

The results are shown in FIGS. **6** and **7**. In this experiment it was confirmed by western blotting (FIG. **6**) and immunostaining (FIG. **7**) that the oligomers PMO Nos. 3 and 8 of the present invention induced expression of the dystrophin protein.

Test Example 5

In Vitro Assay Using Human Fibroblasts

The experiment was performed as in TEST EXAMPLE 3.

Experimental Results

The results are shown in FIG. **8**. This experiment revealed that in the cells from DMD patients with deletion of exons 45-52, the oligomers PMO Nos. 3 to 8 of the present invention caused exon 53 skipping with a higher efficiency than the oligomers PMO Nos. 13 and 14 of the present invention (FIG. **8**).

Test Example 6

In Vitro Assay

Experiments were performed using the antisense oligomers of 2'-O-methoxy-phosphorothioates (2'-OMe-S-RNA) shown by SEQ ID NO: 49 to SEQ ID NO: 123. Various antisense oligomers used for the assay were purchased from Japan Bio Services. The sequences of various antisense oligomers are given below.

TABLE 7

oligomer Antisense	Nucleotide sequence	SEQ ID NO:
H53_39-69	CAUUCAACUGUUGCCUCCGGUUCUGAAGGUG	49
H53_1-25	UCCCACUGAUUCUGAAUUCUUUCAA	50
H53_6-30	CUUCAUCCCACUGAUUCUGAAUUCU	51
H53_11-35	UUGUACUUCAUCCCACUGAUUCUGA	52
H53_16-40	UGUUCUUGUACUUCAUCCCACUGAU	53

TABLE 7 -continued

oligomer Antisense	Nucleotide sequence	SEQ ID NO:
H53_21-45	GAAGGUGUUCUUGUACUUCAUCCCA	54
H53_26-50	GUUCUGAAGGUGUUCUUGUACUUCA	55
H53_31-55	CUCCGGUUCUGAAGGUGUUCUUGUA	56
H53_36-60	GUUGCCUCCGGUUCUGAAGGUGUUC	57
H53_41-65	CAACUGUUGCCUCCGGUUCUGAAGG	58
H53_46-70	UCAUUCAACUGUUGCCUCCGGUUCU	59
H53_51-75	ACAUUUCAUUCAACUGUUGCCUCCG	60
H53_56-80	CUUUAACAUUUCAUUCAACUGUUGC	61
H53_61-85	GAAUCCUUUAACAUUUCAUUCAACU	62
H53_66-90	GUGUUGAAUCCUUUAACAUUUCAUU	63
H53_71-95	CCAUUGUGUUGAAUCCUUUAACAUU	64
H53_76-100	UCCAGCCAUUGUGUUGAAUCCUUUA	65
H53_81-105	UAGCUUCCAGCCAUUGUGUUGAAUC	66
H53_86-110	UUCCUUAGCUUCCAGCCAUUGUGUU	67
H53_91-115	GCUUCUUCCUUAGCUUCCAGCCAUU	68
H53_96-120	GCUCAGCUUCUUCCUUAGCUUCCAG	69
H53_101-125	GACCUGCUCAGCUUCUUCCUUAGCU	70
H53_106-130	CCUAAGACCUGCUCAGCUUCUUCCU	71
H53_111-135	CCUGUCCUAAGACCUGCUCAGCUUC	72
H53_116-140	UCUGGCCUGUCCUAAGACCUGCUCA	73
H53_121-145	UUGGCUCUGGCCUGUCCUAAGACCU	74
H53_126-150	CAAGCUUGGCUCUGGCCUGUCCUAA	75
H53_131-155	UGACUCAAGCUUGGCUCUGGCCUGU	76
H53_136-160	UUCCAUGACUCAAGCUUGGCUCUGG	77
H53_141-165	CCUCCUUCCAUGACUCAAGCUUGGC	78
H53_146-170	GGGACCCUCCUUCCAUGACUCAAGC	79
H53_151-175	GUAUAGGGACCCUCCUUCCAUGACU	80
H53_156-180	CUACUGUAUAGGGACCCUCCUUCCA	81
H53_161-185	UGCAUCUACUGUAUAGGGACCCUCC	82
H53_166-190	UGGAUUGCAUCUACUGUAUAGGGAC	83
H53_171-195	UCUUUUGGAUUGCAUCUACUGUAUA	84
H53_176-200	GAUUUUCUUUUGGAUUGCAUCUACU	85
H53_181-205	UCUGUGAUUUUCUUUUGGAUUGCAU	86
H53_186-210	UGGUUUCUGUGAUUUUCUUUUGGAU	87
H53_84-108	CCUUAGCUUCCAGCCAUUGUGUUGA	88
H53_88-112	UCUUCCUUAGCUUCCAGCCAUUGUG	89
H53_119-143	GGCUCUGGCCUGUCCUAAGACCUGC	90
H53_124-148	AGCUUGGCUCUGGCCUGUCCUAAGA	91
H53_128-152	CUCAAGCUUGGCUCUGGCCUGUCCU	92

TABLE 7 -continued

oligomer Antisense	Nucleotide sequence	SEQ ID NO:
H53_144-168	GACCCUCCUUCCAUGACUCAAGCUU	93
H53_149-173	AUAGGGACCCUCCUUCCAUGACUCA	94
H53_153-177	CUGUAUAGGGACCCUCCUUCCAUGA	95
H53_179-203	UGUGAUUUUCUUUUGGAUUGCAUCU	96
H53_184-208	GUUUCUGUGAUUUUCUUUUGGAUUG	97
H53_188-212	CUUGGUUUCUGUGAUUUUCUUUUGG	98
H53_29-53	CCGGUUCUGAAGGUGUUCUUGUACU	99
H53_30-54	UCCGGUUCUGAAGGUGUUCUUGUAC	100
H53_32-56	CCUCCGGUUCUGAAGGUGUUCUUGU	101
H53_33-57	GCCUCCGGUUCUGAAGGUGUUCUUG	102
H53_34-58	UGCCUCCGGUUCUGAAGGUGUUCUU	103
H53_35-59	UUGCCUCCGGUUCUGAAGGUGUUCU	104
H53_37-61	UGUUGCCUCCGGUUCUGAAGGUGUU	105
H53_38-62	CUGUUGCCUCCGGUUCUGAAGGUGU	106
H53_39-63	ACUGUUGCCUCCGGUUCUGAAGGUG	107
H53_40-64	AACUGUUGCCUCCGGUUCUGAAGGU	108
H53_32-61	UGUUGCCUCCGGUUCUGAAGGUGUUCUUGU	109
H53_32-51	GGUUCUGAAGGUGUUCUUGU	110
H53_35-54	UCCGGUUCUGAAGGUGUUCU	111
H53_37-56	CCUCCGGUUCUGAAGGUGUU	112
H53_40-59	UUGCCUCCGGUUCUGAAGGU	113
H53_42-61	UGUUGCCUCCGGUUCUGAAG	114
H53_32-49	UUCUGAAGGUGUUCUUGU	115
H53_35-52	CGGUUCUGAAGGUGUUCU	116
H53_38-55	CUCCGGUUCUGAAGGUGU	117
H53_41-58	UGCCUCCGGUUCUGAAGG	118
H53_44-61	UGUUGCCUCCGGUUCUGA	119
H53_35-49	UUCUGAAGGUGUUCU	120
H53_40-54	UCCGGUUCUGAAGGU	121
H53_45-59	UUGCCUCCGGUUCUG	122
H53_45-62	CUGUUGCCUCCGGUUCUG	123

RD cells (human rhabdomyosarcoma cell line) were plated at 3×10^5 in a 6-well plate and cultured in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, Inc., hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen Corp.) under conditions of 37° C. and 5% CO_2 overnight. Complexes of various antisense oligomers (Japan Bio Services) (1 μM) for exon 53 skipping and Lipofectamine 2000 (manufactured by Invitrogen Corp.) were prepared and 200 μl was added to RD cells where 1.8 mL of the medium was exchanged, to reach the final concentration of 100 nM.

After completion of the addition, the cells were cultured overnight. The cells were washed twice with PBS (manufactured by Nissui, hereafter the same) and then 500 μl of ISOGEN (manufactured by Nippon Gene) were added to the cells. After the cells were allowed to stand at room temperature for a few minutes for cell lysis, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a Titan One Tube RT-PCR Kit (manufactured by Roche). A reaction solution was prepared in accordance with the protocol attached to the kit. A PTC-100 (manufactured by MJ Research) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

94° C., 2 mins: thermal denaturation

[94° C., 10 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

(SEQ ID NO: 42)
Forward primer: 5'-CATCAAGCAGAAGGCAACAA-3'

(SEQ ID NO: 43)
Reverse primer: 5'-GAAGTTTCAGGGCCAAGTCA-3'

Subsequently, a nested PCR was performed with the amplified product of RT-PCR above using a Taq DNA Polymerase (manufactured by Roche). The PCR program used is as follows.

94° C., 2 mins: thermal denaturation

[94° C., 15 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above are given below.

(SEQ ID NO: 40)
Forward primer: 5'-AGGATTTGGAACAGAGGCGTC-3'

(SEQ ID NO: 41)
Reverse primer: 5'-GTCTGCCACTGGCGGAGGTC-3'

The reaction product, 1 μl, of the nested PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A+B)\times 100$$

Experimental Results

The results are shown in FIGS. **9** to **17**. These experiments revealed that, when the antisense oligomers were designed at exons 31-61 from the 5' end of exon 53 in the human dystrophin gene, exon 53 skipping could be caused with a high efficiency.

Test Example 7

Using an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza), 0.3 to 30 μM of the antisense oligomers

were transfected with 3.5×10^5 of RD cells (human rhabdomyosarcoma cell line). The Program T-030 was used.

After the transfection, the cells were cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, Inc., hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen Corp.) under conditions of 37° C. and 5% CO_2. The cells were washed twice with PBS (manufactured by Nissui, hereinafter the same) and 500 μl of ISOGEN (manufactured by Nippon Gene) was then added to the cells. After the cells were allowed to stand at room temperature for a few minutes to lyse the cells, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit (manufactured by Qiagen, Inc.). A reaction solution was prepared in accordance with the protocol attached to the kit. The thermal cycler used was a PTC-100 (manufactured by MJ Research). The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 30 seconds; 60° C., 30 seconds; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 10 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

(SEQ ID NO: 42)
Forward primer: 5'-CATCAAGCAGAAGGCAACAA-3'

(SEQ ID NO: 43)
Reverse primer: 5'-GAAGTTTCAGGGCCAAGTCA-3'

The reaction product, 1 μl, of the PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)}=A/(A+B)\times100$$

Experimental Results

The results are shown in FIGS. **18** and **19**. These experiments revealed that the oligomer PMO No. 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the antisense oligomers PMO Nos. 15 and 16 (FIG. **18**). It was also revealed that the oligomers PMO Nos. 3 and 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the oligomers PMO Nos. 13 and 14 of the present invention (FIG. **19**). These results showed that the sequences with —OH group at the 5' end provide a higher skipping efficiency even in the same sequences.

INDUSTRIAL APPLICABILITY

Experimental results in TEST EXAMPLES demonstrate that the oligomers of the present invention (PMO Nos. 1 to 10) all caused exon 53 skipping with a markedly high efficiency under all cell environments, as compared to the oligomers (PMO Nos. 11, 12, 15 and 16) in accordance with the prior art. The 5017 cells used in TEST EXAMPLE 2 are the cells isolated from DMD patients, and the fibroblasts used in TEST EXAMPLES 3 and 5 are exon 53 skipping target cells from DMD patients. Particularly in TEST EXAMPLES 3 and 5, the oligomers of the present invention show the exon 53 skipping efficiency of 90% or higher in the cells from DMD patients that are the target for exon 53 skipping. Consequently, the oligomers of the present invention can induce exon 53 skipping with a high efficiency, when DMD patients are administered.

Therefore, the oligomers of the present invention are extremely useful for the treatment of DMD.

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SEQUENCE LISTING

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<211> LENGTH: 19
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<400> SEQUENCE: 26

ccggttctga aggtgttct 19

<210> SEQ ID NO 27
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<212> TYPE: DNA
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<400> SEQUENCE: 27

tccggttctg aaggtgttct 20

<210> SEQ ID NO 28
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<400> SEQUENCE: 28

ctccggttct gaaggtgttc t 21

<210> SEQ ID NO 29
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<212> TYPE: DNA
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cctccggttc tgaaggtgtt ct	22

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<213> ORGANISM: Artificial
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<400> SEQUENCE: 30

gcctccggtt ctgaaggtgt tct	23

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<400> SEQUENCE: 31

tgcctccggt tctgaaggtg ttct	24

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<212> TYPE: DNA
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<400> SEQUENCE: 32

ccggttctga aggtgttc	18

<210> SEQ ID NO 33
<211> LENGTH: 19
<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 33

tccggttctg aaggtgttc	19

<210> SEQ ID NO 34
<211> LENGTH: 20
<212> TYPE: DNA
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<400> SEQUENCE: 34

ctccggttct gaaggtgttc	20

<210> SEQ ID NO 35
<211> LENGTH: 21
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cctccggttc tgaaggtgtt c	21

<210> SEQ ID NO 36
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 36

gcctccggtt ctgaaggtgt tc 22

<210> SEQ ID NO 37
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<400> SEQUENCE: 37

tgcctccggt tctgaaggtg ttc 23

<210> SEQ ID NO 38
<211> LENGTH: 31
<212> TYPE: DNA
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<400> SEQUENCE: 38

cattcaactg ttgcctccgg ttctgaaggt g 31

<210> SEQ ID NO 39
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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 39

ttgcctccgg ttctgaaggt gttcttgtac 30

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<400> SEQUENCE: 40

aggatttgga acagaggcgt c 21

<210> SEQ ID NO 41
<211> LENGTH: 20
<212> TYPE: DNA
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<400> SEQUENCE: 41

gtctgccact ggcggaggtc 20

<210> SEQ ID NO 42
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<212> TYPE: DNA
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catcaagcag aaggcaacaa 20

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<210> SEQ ID NO 43
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<400> SEQUENCE: 43

gaagtttcag ggccaagtca 20

<210> SEQ ID NO 44
<211> LENGTH: 963
<212> TYPE: DNA
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<400> SEQUENCE: 44

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tgctcctttg ccacaacgga cgacttctat gacgacccgt gtttcgactc cccggacctg 120

cgcttcttcg aagacctgga cccgcgcctg atgcacgtgg gcgcgctcct gaaacccgaa 180

gagcactcgc actttccccgc ggcggtgcac ccggcccccgg gcgcacgtga ggacgagcat 240

gtgcgcgcgc ccagcgggca ccaccaggcg ggccgctgcc tactgtgggc ctgcaaggcg 300

tgcaagcgca agaccaccaa cgccgaccgc cgcaaggccg ccaccatgcg cgagcggcgc 360

cgcctgagca aagtaaatga ggcctttgag acactcaagc gctgcacgtc gagcaatcca 420

aaccagcggt tgccccaaggt ggagatcctg cgcaacgcca tccgctatat cgagggcctg 480

caggctctgc tgcgcgacca ggacgccgcg ccccctggcg ccgcagccgc cttctatgcg 540

ccgggcccgc tgccccccggg ccgcggcggc gagcactaca gcggcgactc cgacgcgtcc 600

agcccgcgct ccaactgctc cgacggcatg atggactaca gcggcccccc gagcggcgcc 660

cggcggcgga actgctacga aggcgcctac tacaacgagg cgcccagcga acccaggccc 720

gggaagagtg cggcggtgtc gagcctagac tgcctgtcca gcatcgtgga gcgcatctcc 780

accgagagcc ctgcggcgcc cgccctcctg ctggcggacg tgccttctga gtcgcctccg 840

cgcaggcaag aggctgccgc ccccagcgag ggagagagca gcggcgaccc cacccagtca 900

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tga 963

<210> SEQ ID NO 45
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<212> TYPE: DNA
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<400> SEQUENCE: 45

cgggcttgga cagaacttac 20

<210> SEQ ID NO 46
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 46

tccttacggg tagcatcctg 20

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<210> SEQ ID NO 47
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 47

ctgaaggtgt tcttgtactt catcc 25

<210> SEQ ID NO 48
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 48

tgttgagaaa tggcggcgt 19

<210> SEQ ID NO 49
<211> LENGTH: 31
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 49

cauucaacug uugccuccgg uucugaaggu g 31

<210> SEQ ID NO 50
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 50

ucccacugau ucugaauucu uucaa 25

<210> SEQ ID NO 51
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<212> TYPE: RNA
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<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 51

cuucauccca cugauucuga auucu 25

<210> SEQ ID NO 52
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<212> TYPE: RNA
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<220> FEATURE:
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<400> SEQUENCE: 52

uuguacuuca ucccacugau ucuga 25

<210> SEQ ID NO 53
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 53

uguucuugua cuucauccca cugau 25

<210> SEQ ID NO 54
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<212> TYPE: RNA
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<400> SEQUENCE: 54

gaagguguuc uuguacuuca uccca 25

<210> SEQ ID NO 55
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
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<400> SEQUENCE: 55

guucugaagg uguucuugua cuuca 25

<210> SEQ ID NO 56
<211> LENGTH: 25
<212> TYPE: RNA
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<400> SEQUENCE: 56

cuccgguucu gaagguguuc uugua 25

<210> SEQ ID NO 57
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
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<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 57

guugccuccg guucugaagg uguuc 25

<210> SEQ ID NO 58
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 58

caacuguugc cuccgguucu gaagg 25

<210> SEQ ID NO 59
<211> LENGTH: 25
<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 59

ucauucaacu guugccuccg guucu 25

<210> SEQ ID NO 60
<211> LENGTH: 25

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<212> TYPE: RNA
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<220> FEATURE:
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<400> SEQUENCE: 60

acauuucauu caacuguugc cuccg 25

<210> SEQ ID NO 61
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<212> TYPE: RNA
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<220> FEATURE:
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<400> SEQUENCE: 61

cuuuaacauu ucauucaacu guugc 25

<210> SEQ ID NO 62
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<212> TYPE: RNA
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<400> SEQUENCE: 62

gaauccuuua acauuucauu caacu 25

<210> SEQ ID NO 63
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<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 63

guguugaauc cuuuaacauu ucauu 25

<210> SEQ ID NO 64
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<212> TYPE: RNA
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<400> SEQUENCE: 64

ccauuguguu gaauccuuua acauu 25

<210> SEQ ID NO 65
<211> LENGTH: 25
<212> TYPE: RNA
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<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 65

uccagccauu guguugaauc cuuua 25

<210> SEQ ID NO 66
<211> LENGTH: 25
<212> TYPE: RNA
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<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 66

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uagcuuccag ccauuguguu gaauc 25

<210> SEQ ID NO 67
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 67

uuccuuagcu uccagccauu guguu 25

<210> SEQ ID NO 68
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<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 68

gcuucuuccu uagcuuccag ccauu 25

<210> SEQ ID NO 69
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 69

gcucagcuuc uuccuuagcu uccag 25

<210> SEQ ID NO 70
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 70

gaccugcuca gcuucuuccu uagcu 25

<210> SEQ ID NO 71
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 71

ccuaagaccu gcucagcuuc uuccu 25

<210> SEQ ID NO 72
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 72

ccuguccuaa gaccugcuca gcuuc 25

<210> SEQ ID NO 73
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial

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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 73

ucuggccugu ccuaagaccu gcuca 25

<210> SEQ ID NO 74
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 74

uuggcucugg ccuguccuaa gaccu 25

<210> SEQ ID NO 75
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 75

caagcuuggc ucuggccugu ccuaa 25

<210> SEQ ID NO 76
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 76

ugacucaagc uuggcucugg ccugu 25

<210> SEQ ID NO 77
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 77

uuccaugacu caagcuuggc ucugg 25

<210> SEQ ID NO 78
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 78

ccuccuucca ugacucaagc uuggc 25

<210> SEQ ID NO 79
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 79

gggacccucc uuccaugacu caagc 25

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<210> SEQ ID NO 80
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 80

guauagggac ccuccuucca ugacu 25

<210> SEQ ID NO 81
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 81

cuacuguaua gggacccucc uucca 25

<210> SEQ ID NO 82
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 82

ugcaucuacu guauagggac ccucc 25

<210> SEQ ID NO 83
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 83

uggauugcau cuacuguaua gggac 25

<210> SEQ ID NO 84
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 84

ucuuuuggau ugcaucuacu guaua 25

<210> SEQ ID NO 85
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 85

gauuuucuuu uggauugcau cuacu 25

<210> SEQ ID NO 86
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

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<400> SEQUENCE: 86

ucugugauuu ucuuuuggau ugcau 25

<210> SEQ ID NO 87
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 87

uggauuucugu gauuuucuuu uggau 25

<210> SEQ ID NO 88
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 88

ccuuagcuuc cagccauugu guuga 25

<210> SEQ ID NO 89
<211> LENGTH: 25
<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 89

ucuuccuuag cuuccagcca uugug 25

<210> SEQ ID NO 90
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 90

ggcucuggcc uguccuaaga ccugc 25

<210> SEQ ID NO 91
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 91

agcuuggcuc uggccugucc uaaga 25

<210> SEQ ID NO 92
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 92

cucaagcuug gcucuggccu guccu 25

<210> SEQ ID NO 93

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<211> LENGTH: 25
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<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 93

gacccuccuu ccaugacuca agcuu 25

<210> SEQ ID NO 94
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 94

auagggaccc uccuuccaug acuca 25

<210> SEQ ID NO 95
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 95

cuguauaggg acccuccuuc cauga 25

<210> SEQ ID NO 96
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 96

ugugauuuuc uuuuggauug caucu 25

<210> SEQ ID NO 97
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 97

guuucuguga uuuucuuuug gauug 25

<210> SEQ ID NO 98
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 98

cuugguuucu gugauuuucu uuugg 25

<210> SEQ ID NO 99
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 99

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ccgguucuga agguguucuu guacu 25

<210> SEQ ID NO 100
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 100

uccgguucug aagguguucu uguac 25

<210> SEQ ID NO 101
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 101

ccuccgguuc ugaagguguu cuugu 25

<210> SEQ ID NO 102
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 102

gccuccgguu cugaaggugu ucuug 25

<210> SEQ ID NO 103
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 103

ugccuccggu ucugaaggug uucuu 25

<210> SEQ ID NO 104
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 104

uugccuccgg uucugaaggu guucu 25

<210> SEQ ID NO 105
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 105

uguugccucc gguucugaag guguu 25

<210> SEQ ID NO 106
<211> LENGTH: 25
<212> TYPE: RNA

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<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 106

cuguugccuc cgguucugaa ggugu 25

<210> SEQ ID NO 107
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 107

acuguugccu ccgguucuga aggug 25

<210> SEQ ID NO 108
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 108

aacuguugcc uccgguucug aaggu 25

<210> SEQ ID NO 109
<211> LENGTH: 30
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 109

uguugccucc gguucugaag guguucuugu 30

<210> SEQ ID NO 110
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 110

gguucugaag guguucuugu 20

<210> SEQ ID NO 111
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 111

uccgguucug aagguguucu 20

<210> SEQ ID NO 112
<211> LENGTH: 20
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 112

ccuccgguuc ugaagguguu 20

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cuccgguucu gaaggugu 18

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uguugccucc gguucuga 18

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uucugaaggu guucu 15

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uugccuccgg uucug 15

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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 123

cuguugccuc cgguucug 18

The invention claimed is:

1. An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of the nucleotide sequence of SEQ ID NO: 35, wherein the antisense oligomer is an oligonucleotide having the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide modified, or a morpholino oligomer.

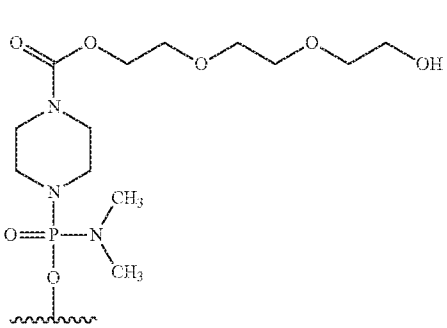
2. The antisense oligomer according to claim **1**, wherein the antisense oligomer is a morpholino oligomer.

3. The antisense oligomer according to claim **1**, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br and I (wherein R is an alkyl or an aryl and R' is an alkylene).

4. The antisense oligomer according to claim **1**, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond and a boranophosphate bond.

5. The antisense oligomer according to claim **2**, wherein the morpholino oligomer is a phosphorodiamidate morpholino oligomer.

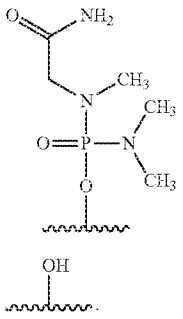
6. The antisense oligomer according to claim **2**, wherein the 5 end of the morpholino oligomer is one of the groups of chemical formulae (1) to (3) below:



(1)

-continued

(2)



(3)

7. A pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active, ingredient the antisense oligomer according to claim **1**, or a pharmaceutically acceptable salt or hydrate thereof.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

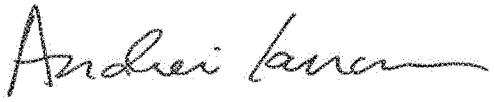
PATENT NO.	: 9,079,934 B2
APPLICATION NO.	: 13/819520
DATED	: July 14, 2015
INVENTOR(S)	: Naoki Watanabe et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Claims

At Column 84, Line 51, replace "the 5 end of" with --the 5' end of--.

Signed and Sealed this
Twenty-third Day of June, 2020



Andrei Iancu
Director of the United States Patent and Trademark Office

ATTACHMENT C

United States Patent and Trademark Office

Office of the Commissioner for Patents

Maintenance Fee Statement

CURRENT MAINTENANCE FEE ADDRESS
CPA GLOBAL LIMITED
2318 MILL ROAD 12TH FLOOR
ALEXANDRIA, VA 22314

CUSTOMER #
197

ENTITY STATUS
UNDISCOUNTED

STATEMENT GENERATED
09/20/2020 21:55:29

Invention

ANTISENSE NUCLEIC ACIDS

PATENT #	APPLICATION #	FILING DATE	ISSUE DATE
9079934	13819520	04/10/2013	07/14/2015

Payment Details

PAYMENT DATE	DATE POSTED	TRANSACTION ID	ATTORNEY DOCKET #	TOTAL PAYMENT
01/03/2019	01/03/2019	010319INTMTFEE00007950504623		$1,600.00

Fee Code	Description	Safe ID	Fee Amount
1551	MAINTENANCE FEE DUE AT 3.5 YEARS	010319INTMTFEE00007950	$1,600.00

According to the records of the United States Patent and Trademark Office (USPTO), the maintenance fee and any necessary surcharge have been timely paid for the patent listed above. The payment shown above is subject to actual collection. If the payment is refused or charged back by a financial institution, the payment will be void and the maintenance fee and any necessary surcharge unpaid.

ATTACHMENT D

Attachment D

Summary of Events VILTEPSO™ (viltolarsen)

Date[1]	Initiated By	Type	Brief Description
20150728	NSP[2]	Email	Question for pre-IND Meeting planning
20150805	NSP	Email	pre-IND meeting request
20150824	NSP	Email	pre-IND meeting request granted
20150918	NSP	Mail	pre-IND meeting information package
20151006	NSP	Email	Questions on pre-IND meeting & breakthrough therapy designation; FDA responded
20151019	NSP	Email	pre-IND meeting - Division preliminary comments & sponsor responses
20151020	NSP	Meeting	pre-IND meeting
20160325	NSP	eCTD	Submit original IND to FDA

[1] The date is provided as year, month, date.

[2] "NSP" refers to a US agent of the NDA applicant and holder, NS Pharma, Inc., which is an affiliate of the patent holder and NDA applicant and holder.

Date[1]	Initiated By	Type	Brief Description
20160328	NSP	Email	Clarification/request for point of contact for the IND
20160328	FDA	Email	IND acknowledgement by the FDA
20160407	FDA	Email	RFI[3] on clinical question during IND review
20160411	NSP	Email	Response to 7-Apr-16 clinical RFI
20160423	FDA	Email	Email confirmation from FDA that study may proceed (in absence of official letter)
20160504	FDA	Email	Official letter that IND study may proceed
20160513	NSP	eCTD	Submit update to Form 3674 (NCT #) & sponsor response to RFI
20160606	NSP	Email	Question on DSUR and DIBD request[4]
20160803	NSP	Email	Question on safety reporting
20160902	NSP	Email	Questions on planned IND amendments,

[3] "RFI" stands for Request for Information.

[4] "DSUR" stands for development safety update report and "DIBD" stands for development international birth date.

Date[1]	Initiated By	Type	Brief Description
			including fast track
20161011	NSP	eCTD	Submit fast track designation request
20161027	FDA	Email/Mail	Letter granting fast track designation
20161118	NSP	eCTD	Submit Study 201 Protocol A2 and request comment (SN0003)
20161130	NSP	Mail	Submit rare pediatric disease designation (RPDD) request
20161130	NSP	Mail	Submit orphan drug designation (ODD) request
20161216	NSP	eCTD	Study 201 investigator information
20170112	FDA	Email/Mail	FDA letter granting ODD
20170124	FDA	Email	FDA provided feedback to the SN0003 request for comment on tox study questions
20170124	FDA	Mail	FDA issued letter granting RPDD
20170206	NSP	eCTD	Submit request for change in DSUR-AR[5] due

[5] "AR" stands for Annual Report.

Date[1]	Initiated By	Type	Brief Description
			date & submission of ODD and RPDD granted letters
20170307	FDA	Email	Receive DSUR-AR revised date granted letter
20170316	FDA	Email	Response to the SN0003 request for comment on tox study question
20170327	NSP	Email	Provide response to FDA comment on tox study
20170328	FDA	Email	FDA comments on NSP questions on tox study from request for comment (SN0003)
20170420	NSP	eCTD	Type C meeting request for bioanalytical methods; NSP submits questions on tox study
20170501	FDA	Email	Type C meeting request granted letter
20170526	NSP	eCTD	Type C meeting information package
20170629	NSP	eCTD	Study 201 Protocol A4 and Study 202 Protocol A1 with investigator information
20170705	FDA	Email	Type C WR (written response)

Date[1]	Initiated By	Type	Brief Description
20170707	FDA	Email	FDA information request for nondinical study pathology statements
20170714	NSP	Email	Response to FDA's request for information on pathology statements
20170728	NSP	Email	Delivery of nonclinical study pathology statements in response to FDA request
20170802	NSP	eCTD	Submit nonclinical study pathology statement in response to FDA information request
20170804	NSP	eCTD	Request for comment on DP (drug product) stability & Type C WR (SN0013)
20170808	FDA	Email	Request to submit request for comment on DP stability (SN0013) as Type C WRO request
20170814	NSP	eCTD	Submit Type C WRO request for DP stability
20170821	NSP	eCTD	Submit Sponsor response to Type C WR
20170823	FDA	Email	FDA comments on sponsor questions for

Date[1]	Initiated By	Type	Brief Description
			clarification (Type C WR)
20170908	NSP	Email	Follow-up sponsor responses to FDA email feedback
20170920	NSP	eCTD	Submit Type C WRO information package
20171012	FDA	Email	Type C WR (DP stability)
20171020	NSP	eCTD	Submit Module 3 update with DP
20171024	NSP	eCTD	Submit Study 202 Protocol A2 with investigator information
20171221	NSP	eCTD	Submit Study 201 Protocol A5
20171221	NSP	eCTD	Study 201 Protocol A6 and Type C WR (DP stability)
20180123	NSP	eCTD	Submit Study 202 Protocol A3
20180123	NSP	eCTD	Submit Study 202 Protocol A4
20180215	NSP	eCTD	Submit Module 3 update for new strength
20180220	NSP	eCTD	Submit breakthrough therapy designation (BTD)

Date[1]	Initiated By	Type	Brief Description
			request
20180222	FDA	Email	Acknowledgement of BTD request
20180227	NSP	eCTD	Submit Type C meeting request for clinical & CMC[6]
20180306	FDA	Email	Notice of new FDA PM[7]
20180309	FDA	Email	Type C meeting request granted letter
20180320	NSP	Email	Request if 2-Apr-18 receipt of Type C information package desk copies is acceptable; FDA responded
20180327	NSP	Email	Notification inquiry of carcinogenicity SPA[8] request in mid/late May
20180327	NSP	eCTD	Submit Type C meeting information package for clinical & CMC

[6] "CMC" stands for Chemistry Manufacturing and Controls.

[7] "PM" stands for project manager.

[8] "SPA" stands for Special Protocol Assessment.

Date[1]	Initiated By	Type	Brief Description
20180427	NSP	Email	Notification of NSP intent to submit carcinogenicity SPA
20180501	NSP	Email	Delivery of Type C meeting data supplement
20180503	NSP	eCTD	Submit data supplement to Type C meeting information package for clinical & CMC
20180509	FDA	Email	Delivery of FDA preliminary comments to Type C meeting information package
20180514	NSP	Email	Delivery of sponsor responses to the FDA's preliminary comments
20180514	FDA	Email	FDA inquiry about status of NSP's plans/questions for the Type C meeting
20180515	NSP	Meeting	Type C meeting
20180515	NSP	Email	Pre-assigned eCTD NDA number request
20180516	FDA	Email	Assignment of NDA number
20180517	NSP	eCTD	Submit request for proprietary name review

Date[1]	Initiated By	Type	Brief Description
20180601	NSP	eCTD	Submit request for carcinogenicity SPA (SN0029)
20180604	NSP	Email	Deliver copy of SN0029 cover letter for request for SPA
20180605	FDA	Email	FDA provides acknowledgement of receipt for SPA request
20180713	NSP	eCTD	Submit DSUR[9]
20180720	NSP	eCTD	Submit pre-NDA meeting request
20180724	FDA	Email	FDA information request (bioanalytical methods)
20180730	NSP	eCTD	Submit Protocol 202 A5
20180730	NSP	eCTD	Submit Protocol 202 A5.1
20180730	NSP	eCTD	Submit Protocol 202 A6 & ICF[10] version 6
20180730	NSP	eCTD	Submit Protocol 202 A7 & ICF version 7

[9] "DSUR" stands for development safety update report.

[10] "ICF" stands for informed consent form.

Date[1]	Initiated By	Type	Brief Description
20180801	FDA	Email	pre-NDA meeting granted letter
20180817	NSP	eCTD	Submit bioanalytical methods & nonclinical bridging study reports
20180822	NSP	eCTD	Submit pre-NDA meeting information package
20180913	FDA	Email	Reviewer comment and recommendation on bioanalytical methods
20180917	NSP	Email	Sponsor inquiry on date for FDA internal meeting & response to reviewer's comments on bioanalytical methods
20180919	FDA	Email	FDA clarification on 13-Sep-18 comment
20180926	NSP	Meeting	pre-NDA meeting
20181108	NSP	eCTD	Submit nonclinical information amendment
20181113	FDA	Email	Request for update on rolling review submission schedule
20181120	NSP	eCTD	Submit clinical information amendment -

Date[1]	Initiated By	Type	Brief Description
			including request for comments/advice on revised confirmatory study synopsis
20181128	NSP	eCTD	Submit rolling review request with updated NDA submission schedule
20181203	NSP	Email	Request for status update on open items at pre-NDA meeting; FDA responded
20181203	FDA	Email	Receipt of acknowledgement of rolling review request
20181214	NSP	Email	Status update follow-up to 3-Dec-18 email correspondence on open items
20181220	FDA	Email	FDA response to 14-Dec-18 status update follow-up
20190108	NSP	eCTD	Submit nonclinical information amendment & 20-Dec-18 FDA correspondence
20190114	NSP	Email	Request status of rolling review request

Date[1]	Initiated By	Type	Brief Description
20190115	FDA	Email	Grant rolling review for NDA 212154
20190201	NSP	eCTD	Submit NDA Wave 1 (nonclinical)
20190226	FDA	Email	Acknowledgement of pre-submission letter
20190610	FDA	Email	Information request - inspection readiness & submission timing
20190617	NSP	Email	Response to 10-Jun-19 division information request
20190618	FDA	Email	FDA agreement on plan for NDA nonclinical information amendment
20190627	NSP	eCTD	Submit Phase 3 (Study 301) protocol, ICF, and SAP[11]
20190628	NSP	eCTD	Rat carcinogenicity SPA request
20190708	NSP	eCTD	DSUR (IND annual report)
20190813	NSP	Email	NDA status update and request for agreement

[11] "SAP" stands for Statistical Analysis Plan.

Date[1]	Initiated By	Type	Brief Description
			on image data, safety update report
20190820	FDA	Email	FDA comment on Phase 3 study submitted on 27-June-19
20190820	NSP	eCTD	Submit request for comment and advice
20190822	FDA	Email	FDA responses to 13-Aug-19 email (image data)
20190827	FDA	Email	Completed FDA responses to 13-Aug-19 email (safety update report)
20190918	FDA	Email	Request for status of NDA 212154 submission completion
20190920	NSP	eCTD	Submit IND CMC amendment for Phase 3 study
20190926	NSP	eCTD	Submit IND amendment for Study 201/202 - change in investigators
20190927	NSP	eCTD	Submit NDA Wave 2 (clinical and quality)
20190930	FDA	Email	Request for clarification on relationship

Date[1]	Initiated By	Type	Brief Description
			between NSP and NS HQ[12]
20191007	FDA	Email	Courtesy copy of US NDA acknowledgement Letter
20191008	FDA	Email	Clinical information request
20191018	NSP	eCTD	Submit response to 8-Oct-19 clinical information request
20191022	FDA	Email	Clinical information request
20191023	FDA	Email	Follow-up clinical information request
20191025	NSP	eCTD	Submit proprietary name review request
20191030	NSP	eCTD	Submit response to 23-Oct-19 clinical information request
20191030	NSP	eCTD	Submit nonclinical information amendment - multimer studies
20191101	NSP	eCTD	Submit response to 22-Oct-19 clinical

[12] "NS HQ" is Nippon Shinyaku Head Quarters, the NDA applicant and holder.

Date[1]	Initiated By	Type	Brief Description
			information request
20191107	NSP	Email	Agreement to continue rolling submission
20191114	NSP	Email	Delivery of method validation plan; FDA responded on 15-Nov-19 and accepted on 18-Nov-19
20191115	NSP	eCTD	Submit Protocol 301 Amendment - version 1.1 (ICFs)
20191119	NSP	eCTD	Submit Protocol 301 Amendment - version 1.2 (ICFs)
20191121	NSP	eCTD	Submit 2nd response to 22-Oct-19 information request – clinical information amendment with method validation plan
20191122	NSP	Email	Notification of applicant intention to submit safety update report year-end
20191122	NSP	eCTD	Submit Study 301 new investigator & financial

Date[1]	Initiated By	Type	Brief Description
			certification
20191125	NSP	Meeting	Informal tcon on request for expedited filing
20191126	NSP	Email	Email summarizing 25-Nov-19 request expedited filing
20191203	NSP	eCTD	Submit Protocol 202 A8 (with ICF revisions)
20191204	NSP	Email	Status of method validation & 26-Nov-19 expedite NDA filing request; FDA responded
20191210	NSP	eCTD	Submit request for comment - Study 211
20191211	FDA	Email	Clinical information request
20191211	NSP	Email	Notification of submission of method validation report; FDA responded
20191212	NSP	eCTD	Submit 3rd response to 22-Oct-19 clinical information request
20191214	NSP	eCTD	Submit response to 11-Dec-19 information request

Date[1]	Initiated By	Type	Brief Description
20191231	NSP	eCTD	Submit NDA safety update report
20200107	FDA	Email	Receive NDA 212154 acknowledgement letter
20200107	NSP	Email	NSP acknowledges receipt of acknowledgment letter
20200110	FDA	Email	Proprietary name request unacceptable /disclosure authorization request
20200113	FDA	Email	Clinical information request: QT risk assessment
20200114	NSP	Email	NSP question about purpose of 13-Jan-20 information request; FDA responded
20200115	NSP	Email	NSP informs FDA of timing of 13-Jan-20 information request submission
20200115	FDA	Email	Acknowledge 13-Jan-20 information request submission timing
20200117	NSP	Email	Inform FDA that there is delay in providing response

Date[1]	Initiated By	Type	Brief Description
20200117	FDA	Email	Acknowledge 13-Jan-20 information request submission timing
20200120	NSP	Email	Submit 13-Jan-20 information request response via email
20200122	FDA	Email	Request for formal submission of 13-Jan-20 information request
20200122	NSP	eCTD	Submit response to 13-Jan-20 information request on QT risk assessment
20200122	FDA	Email	Request to secure email and submit disclosure authorization officially to NDA
20200122	NSP	eCTD	Submit response for proprietary name/response to disclosure authorization request
20200124	FDA	Email	Copy of fax about 22-Jan-20 communication
20200124	FDA	Email	Clinical information request
20200124	FDA	Email	Response to question about disclosure

Date[1]	Initiated By	Type	Brief Description
			information
20200127	NSP	Email	Response to 24-Jan-20 information request
20200128	FDA	Email	CMC information request
20200130	NSP	Email	NSP question about timing of acceptance to file and Day 74 letter
20200204	FDA	Email	Notification that agency is preparing officially response regarding proprietary name
20200205	NSP	Email	Update on carcinogenicity study to FDA
20200206	FDA	Email	No filing review issues identified
20200207	NSP	eCTD	Submit Study 301 new investigators
20200210	NSP	Email	NSP request for extension on 28-Jan-20 information request
20200210	NSP	Email	NSP question about if we can send revised draft PI (Package Insert)
20200211	FDA	Email	FDA grants extension on 28-Jan-20 information

Date[1]	Initiated By	Type	Brief Description
			request timing
20200214	FDA	Email	Clinical information request: bioanalytical methods
20200214	NSP	eCTD	Submit response to 28-Jan-20 information request on CMC
20200218	NSP	Email	NSP informing FDA that CMC information request was submitted through the ESG[13]
20200221	NSP	Email	NSP email response to FDA for 14-Feb-20 information request
20200226	NSP	Email	NSP question about Day 74 letter; FDA responded
20200226	NSP	eCTD	Submit response to 14-Feb-20 information request
20200305	FDA	Email	Request for status of carcinogenicity study

[13] "ESG" stands for electronic submissions gateway.

Date[1]	Initiated By	Type	Brief Description
20200306	NSP	Email	NSP responds to FDA request for status of carcinogenicity study
20200306	FDA	Email	FDA requests draft reports on carcinogenicity study
20200306	FDA	Email	CMC information request: DP
20200309	FDA	Email	FDA informs NSP of date of mid-cycle communication meeting
20200309	NSP	Email	NSP provides documents regarding carcinogenicity study
20200310	FDA	Email	CMC information request: DS (drug substance)
20200311	NSP	eCTD	Submit Protocol 202 A9, updated ICF and IB addendum
20200317	NSP	eCTD	Submit Protocol 301 A3, updated ICF & assent form
20200318	FDA	Email	CMC information request: DP

Date[1]	Initiated By	Type	Brief Description
20200319	NSP	eCTD	Submit Study 301 new investigators
20200320	NSP	eCTD	Submit CMC information amendment: 10-Mar-20 information request
20200323	FDA	Email	Clinical information request: dosing procedure & exposure data
20200325	-	Meeting	Mid-cycle communication meeting
20200325	NSP	eCTD	Submit CMC information amendment: 6-Mar-20 information request
20200327	NSP	eCTD	Submit clinical information amendment: 23-Mar-20 information request
20200331	FDA	Email	CMC information request: container and carton labeling
20200401	NSP	eCTD	Submit CMC information amendment: 18-Mar-20 information request
20200402	FDA	Email	Clinical information request: data file for Study

Date[1]	Initiated By	Type	Brief Description
			201
20200402	FDA	Email	Clinical information request: study manuals
20200406	FDA	Email	Clinical information request
20200407	NSP	eCTD	Submit clinical information amendment: 2-Apr-20 information request on study manuals
20200407	NSP	eCTD	Submit CMC information amendment: 31-Mar-20 information request
20200409	FDA	Email	CMC Information request: DS
20200409	NSP	eCTD	Submit CMC amendment (supporting EAP)[14]
20200409	NSP	eCTD	Submit clinical information amendment: 2-Apr-20 information request (revised study 201 dataset)
20200410	NSP	eCTD	Submit clinical information amendment: 6-Apr-20 information request

[14] "EAP" stands for expanded access program.

Date[1]	Initiated By	Type	Brief Description
20200410	NSP	eCTD	Submit clinical information amendment: 20-Mar-20 information request (mid-cycle communication)
20200414	FDA	Email	CMC Information request
20200416	NSP	eCTD	Submit CMC information amendment: 9-Apr-20 information request
20200417	FDA	Email	Clinical information request
20200420	NSP	Email	Sponsor asks for updates to proprietary name
20200422	NSP	eCTD	Submit CMC information amendment: 14-Apr-20 information request
20200424	NSP	eCTD	Submit clinical information amendment: 17-Apr-20 information request
20200424	FDA	Email	Summary of call regarding OPDP (The Office of Prescription Drug Promotion) questions
20200427	FDA	Email	Clinical information request: P3 study status

Date[1]	Initiated By	Type	Brief Description
20200427	FDA	Email	CMC Information request: manufacturer testing
20200429	NSP	eCTD	Submit change in regulatory contact
20200429	NSP	eCTD	Submit clinical information amendment: 27-Apr-20 information request
20200430	NSP	eCTD	Submit CMC information amendment: 27-Apr-20 information request
20200520	FDA	Email	FDA advice/information request on Study 301
20200520	NSP	eCTD	Submit clinical information amendment: 4-May-20 request for Study 301 status
20200529	NSP	eCTD	Submit CMC information amendment: USP compliance statements (resolve 14-Apr-20 information request)
20200604	NSP	eCTD	Request for proprietary name review
20200605	NSP	eCTD	Submit applicant response to late-cycle meeting background package

Date[1]	Initiated By	Type	Brief Description
20200608	NSP	eCTD	Submit update to proprietary name review request
20200610	-	Meeting	Late-cycle meeting
20200611	FDA	Email	Submission of ECG datasets with a formal request for a TQT waiver to NDA 212154[15]
20200615	FDA	Email	Proprietary name request for Viltepso conditionally acceptable
20200619	NSP	eCTD	Submit response to 20-May-20 FDA advice/information request on Study 301 (SN0060)
20200626	NSP	Email	Follow up questions on status of review of SN0060
20200629	NSP	eCTD	Submit Study 301 new investigators
20200710	NSP	eCTD	Submit IND annual report (DSUR format)

[15] "ECG" stands for electrocardiogram. "TQT" stands for Through QT.

Date[1]	Initiated By	Type	Brief Description
20200716	FDA	Email	PMRs 1-4, general timeline inquiries[16]
20200719	NSP	Email	Feedback requested on future commitment for additional CCIT[17]
20200722	NSP	eCTD	Submit CMC information amendment: CCIT report in response to 28-Jan-20 information request
20200723	NSP	Email	Follow up questions on status of review of SN0060 (19-Jun-20)
20200724	FDA	Email	Immunogenicity PMR timeline
20200727	FDA	Email	FDA follow-up comments on Protocol 301 A2 (SN0060)
20200727	NSP	Email	NSP requested teleconference with the FDA if clarification is needed on questions & comments of SN0060

[16] "PMR" stands for post-marketing requirements.

[17] "CCIT" stands for container closure integrity testing.

Date[1]	Initiated By	Type	Brief Description
20200727	FDA/NSP	Email	Labeling comments & follow-up
20200728	FDA	Email	Email correspondence on revised carton & container labeling
20200728	NSP	eCTD	Submit carton & container labeling update
20200729	NSP	Email	NSP adds clarification comment on immunogenicity PMR
20200730	NSP	Email	NSP requests clarification comments on PMRs 1-4
20200804	FDA	Email	2-year rat carcinogenicity study PMR
20200804	FDA/NSP	Email	Labeling comments & follow up (Round 3)
20200807	NSP	eCTD	Submit MedWatch Form for an Initial Report of suspected unexpected serious adverse reactions (SUSAR)
20200810	NSP	eCTD	Submit final PMR
20200810	FDA/NSP	Email	Labeling comments & follow up (Round 4)

Date[1]	Initiated By	Type	Brief Description
20200811	FDA/NSP	Email	Labeling comments & follow up (Round 5)
20200812	FDA	Email	Approval of NDA 212154 for VILTEPSO™ (viltolarsen)
20200818	NSP	eCTD	Submit MedWatch follow-up submission
20200821	NSP	eCTD	Submit Protocol 202 amendment 10 (v11) and ICF v10
20200911	NSP	Email	Review status update from FDA on SN0060

EXHIBIT AC

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

August 27, 2021

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THIS OFFICE OF:

U.S. PATENT: *9,708,361*

ISSUE DATE: *July 18, 2017*

By Authority of the

Under Secretary of Commerce for Intellectual Property

and Director of the United States Patent and Trademark Office

T. WALLACE

Certifying Officer

EXHIBIT

Watanabe #2

tabbies

(12) **United States Patent**
Watanabe et al.

(10) **Patent No.: US 9,708,361 B2**
(45) **Date of Patent: Jul. 18, 2017**

(54) **ANTISENSE NUCLEIC ACIDS**

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(72) Inventors: **Naoki Watanabe**, Tsukuba (JP); **Youhei Satou**, Tsukuba (JP); **Shin'ichi Takeda**, Kodaira (JP); **Tetsuya Nagata**, Kodaira (JP)

(73) Assignees: **NIPPON SHINYAKU CO., LTD.**, Kyoto-shi, Kyoto (JP); **NATIONAL CENTER OF NEUROLOGY AND PSYCHIATRY**, Tokyo (JP)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **14/615,504**

(22) Filed: **Feb. 6, 2015**

(65) **Prior Publication Data**

US 2015/0166995 A1 Jun. 18, 2015

Related U.S. Application Data

(63) Continuation of application No. 13/819,520, filed as application No. PCT/JP2011/070318 on Aug. 31, 2011, now Pat. No. 9,079,934.

(30) **Foreign Application Priority Data**

Sep. 1, 2010 (JP) 2010-196032

(51) **Int. Cl.**

C07H 21/02	(2006.01)
C07H 21/04	(2006.01)
A61K 31/70	(2006.01)
C12N 15/11	(2006.01)
C12N 15/113	(2010.01)
C07H 21/00	(2006.01)
C12N 5/00	(2006.01)

(52) **U.S. Cl.**

CPC ***C07H 21/04*** (2013.01); ***C07H 21/00*** (2013.01); ***C12N 15/111*** (2013.01); ***C12N 15/113*** (2013.01); *C12N 2310/11* (2013.01); *C12N 2310/315* (2013.01); *C12N 2310/3145* (2013.01); *C12N 2310/321* (2013.01); *C12N 2310/3525* (2013.01); *C12N 2320/33* (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

(56) **References Cited**

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Primary Examiner — Sean McGarry

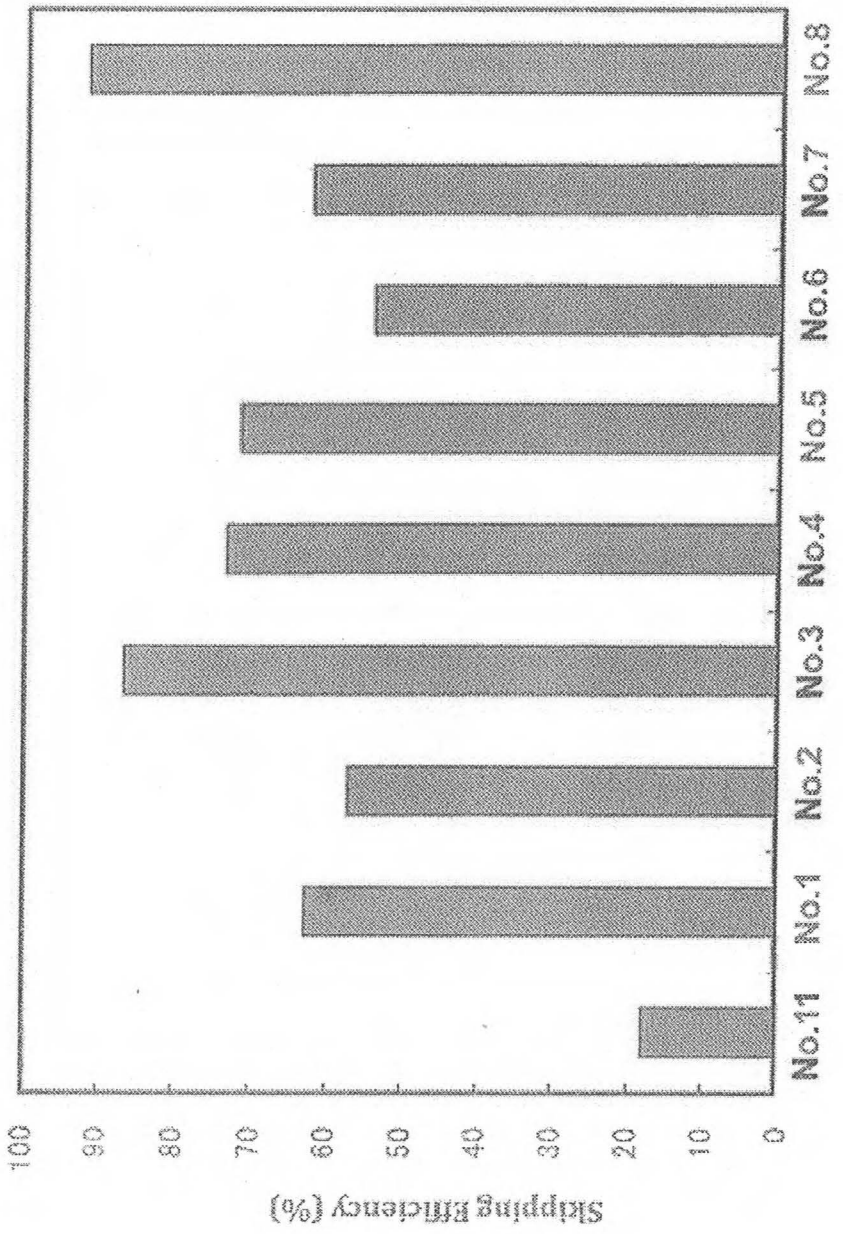
(74) *Attorney, Agent, or Firm* — Drinker Biddle & Reath LLP

(57) **ABSTRACT**

The present invention provides an oligomer which efficiently enables to cause skipping of the 53rd exon in the human dystrophin gene. Also provided is a pharmaceutical composition which causes skipping of the 53rd exon in the human dystrophin gene with a high efficiency.

7 Claims, 19 Drawing Sheets

Figure 1



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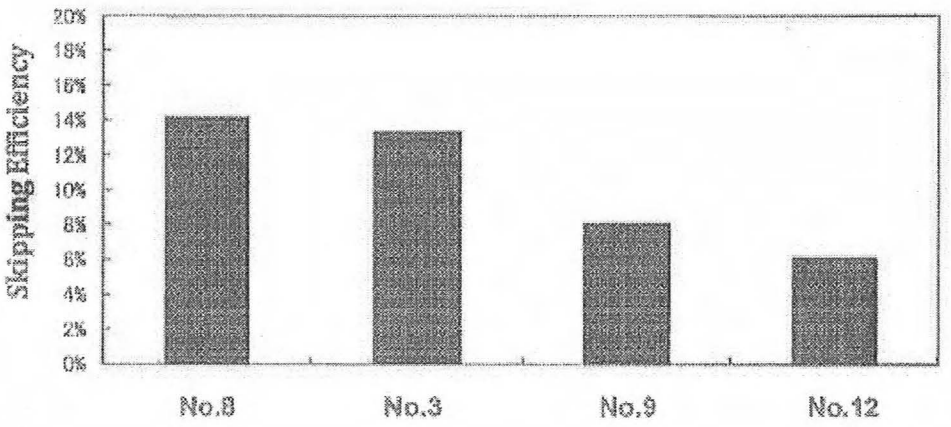

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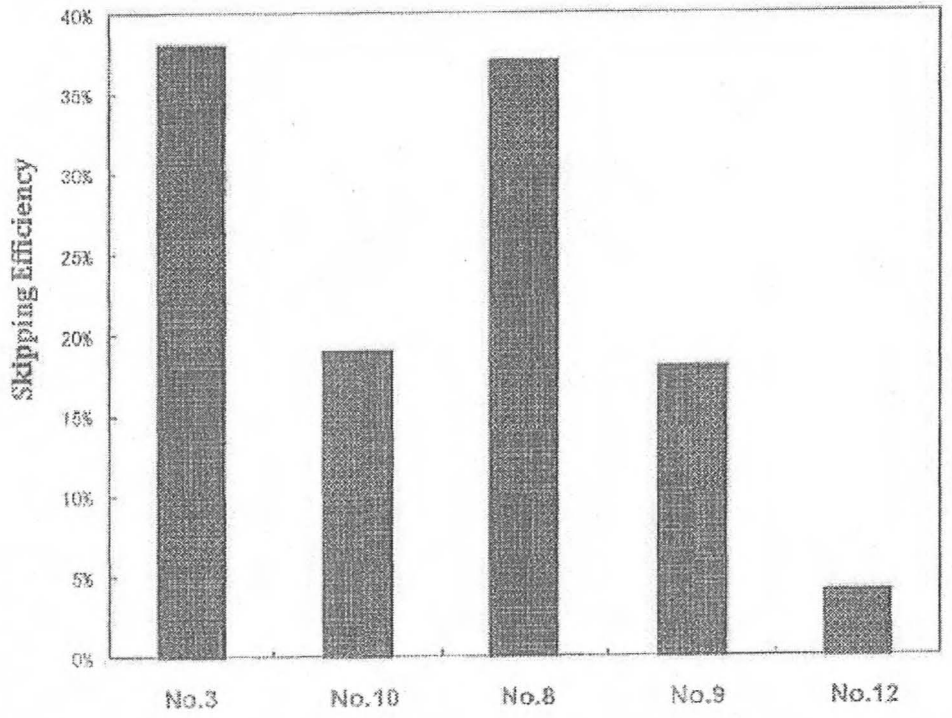


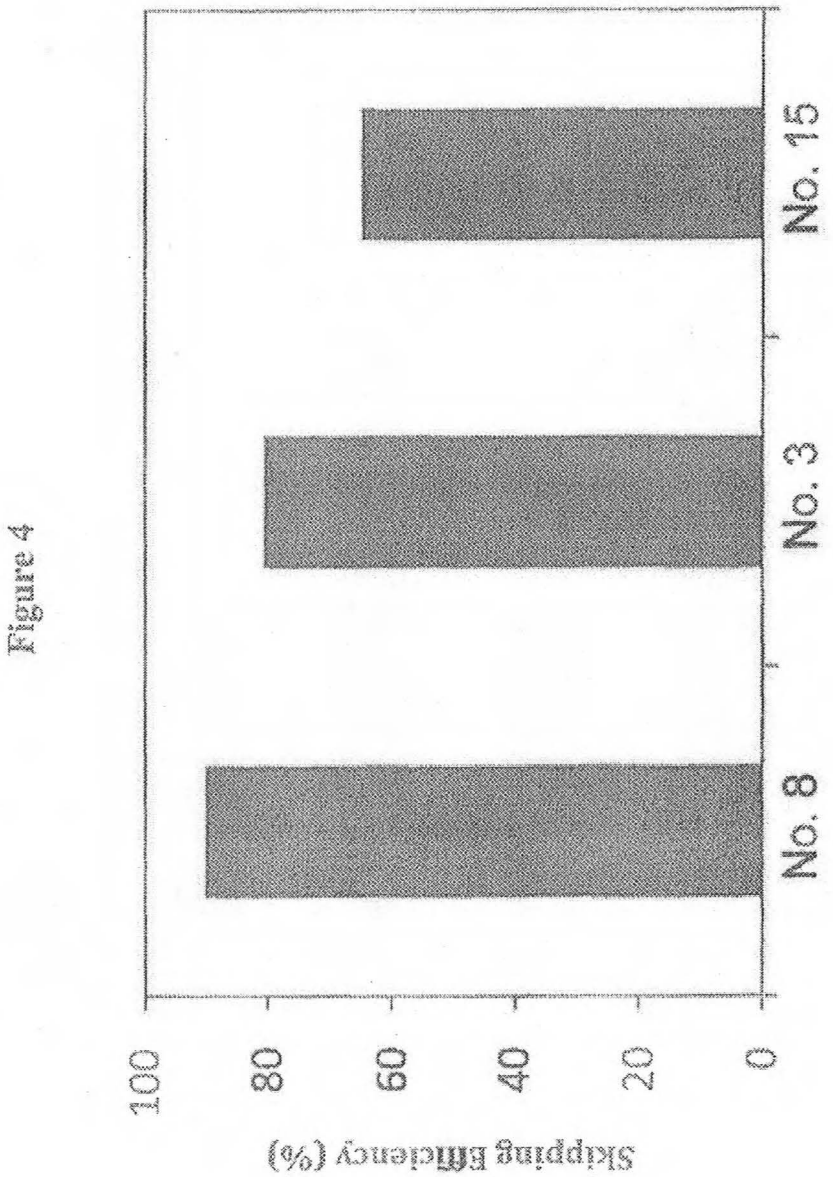
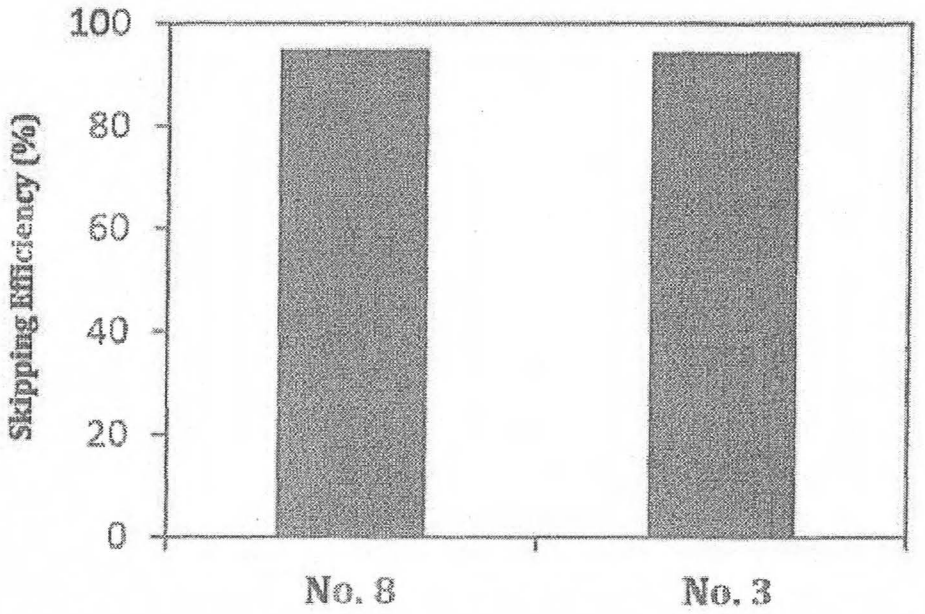


Figure 4

Figure 5



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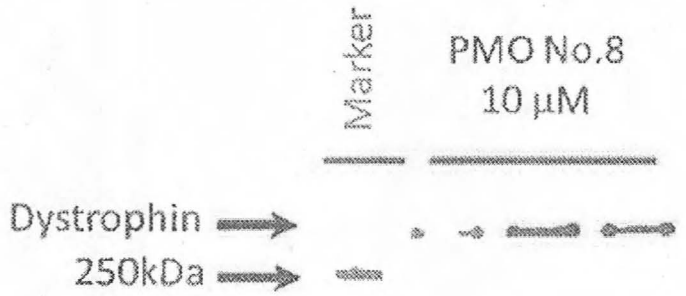

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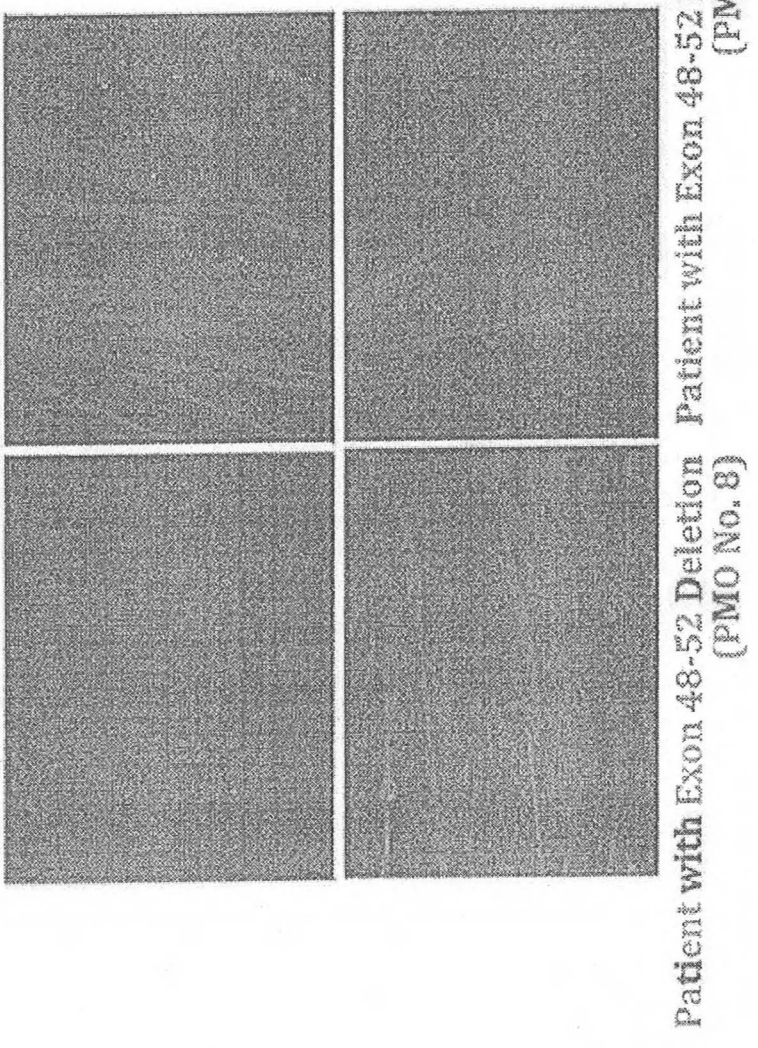

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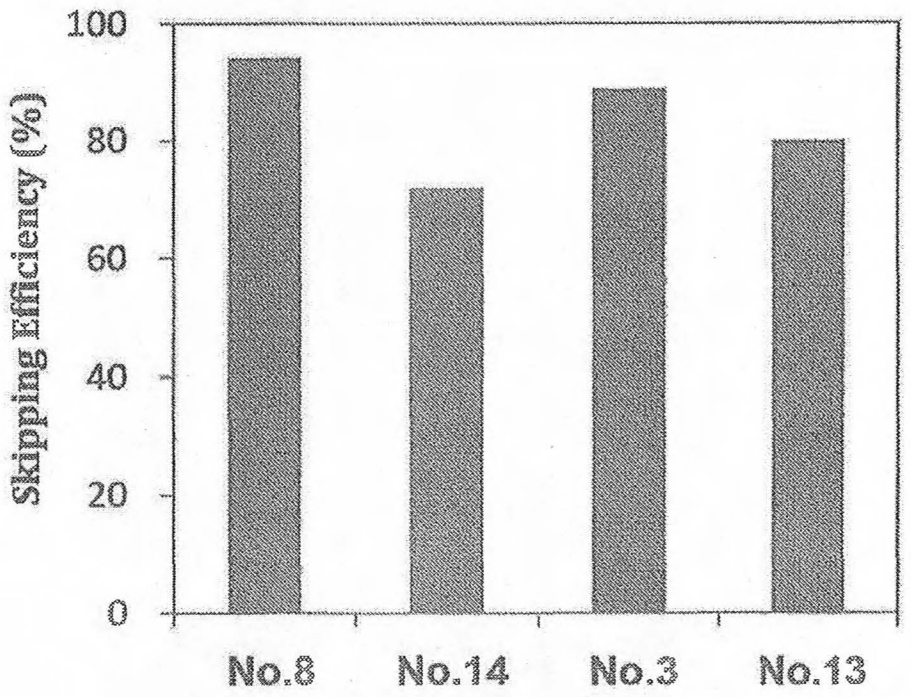

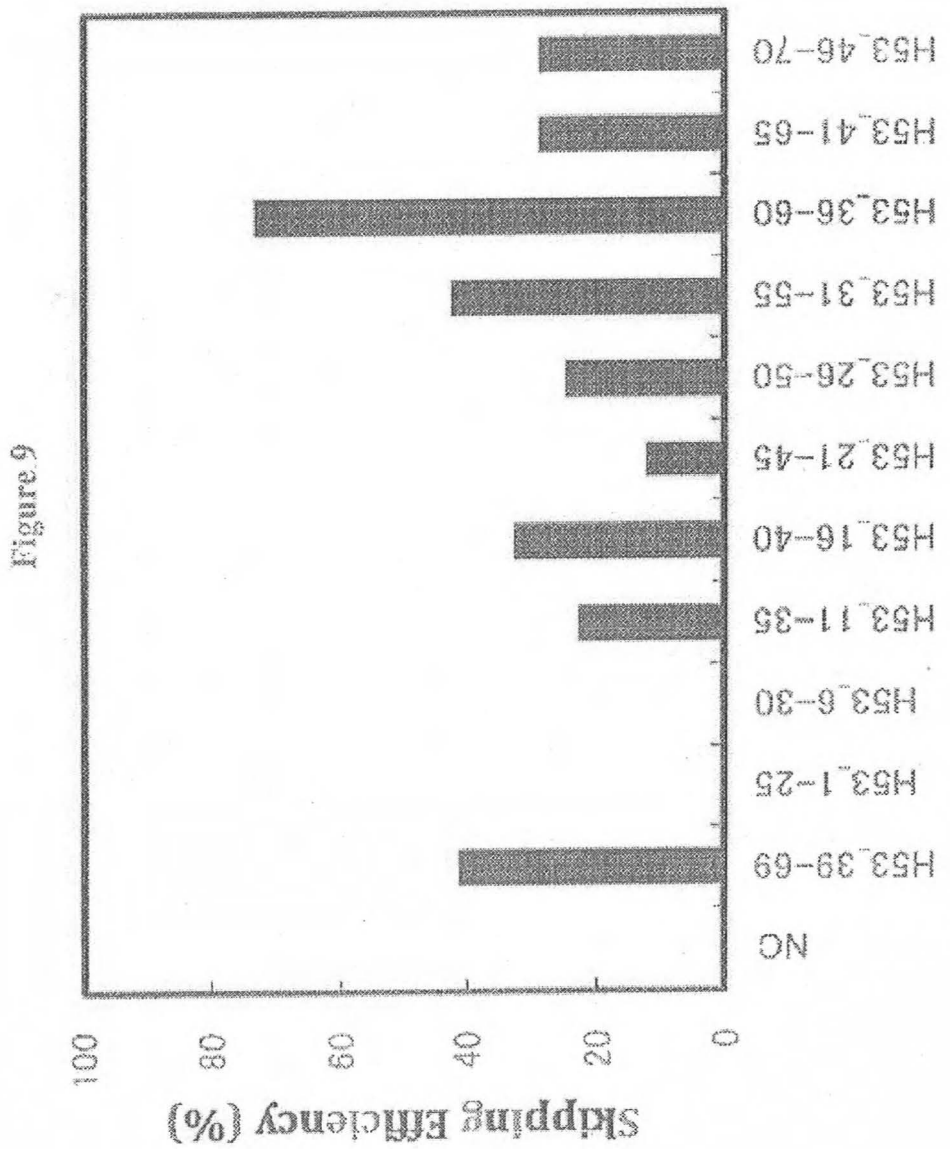

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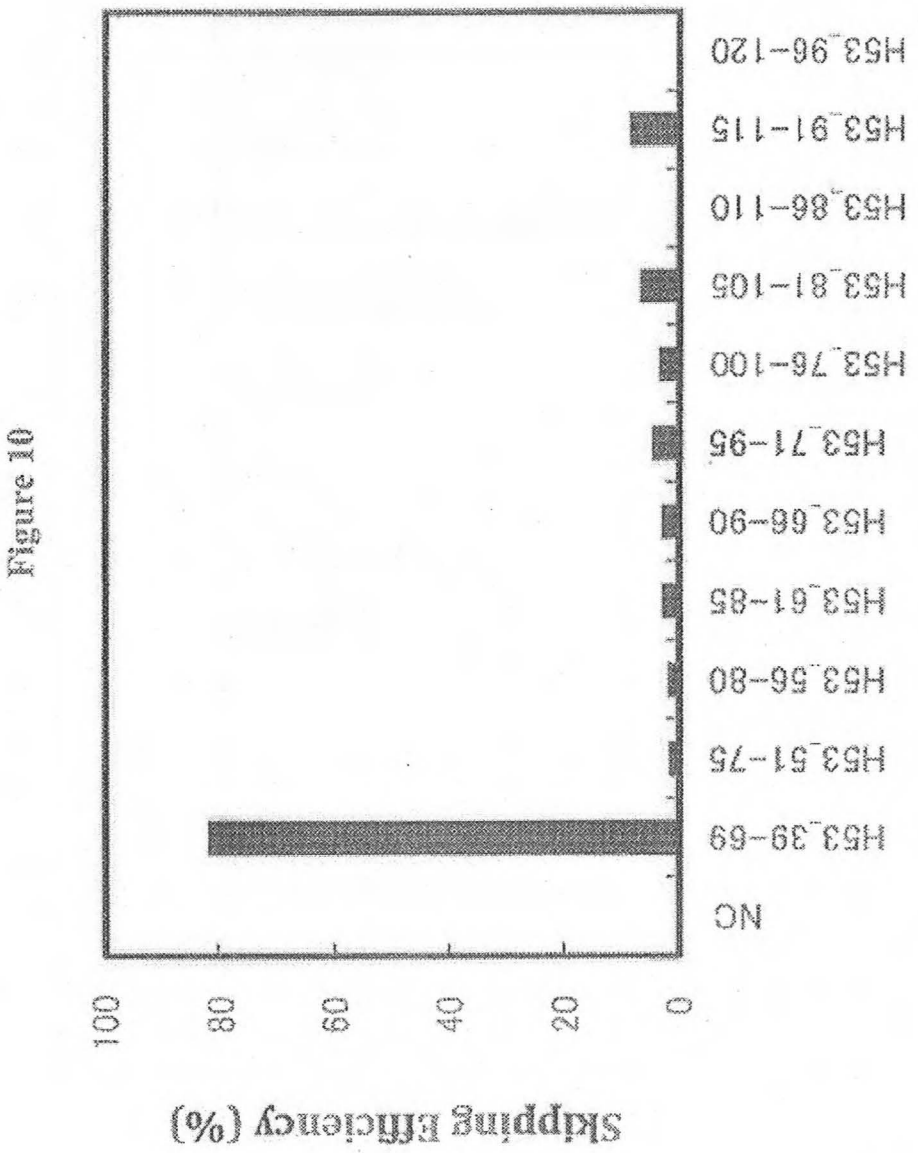

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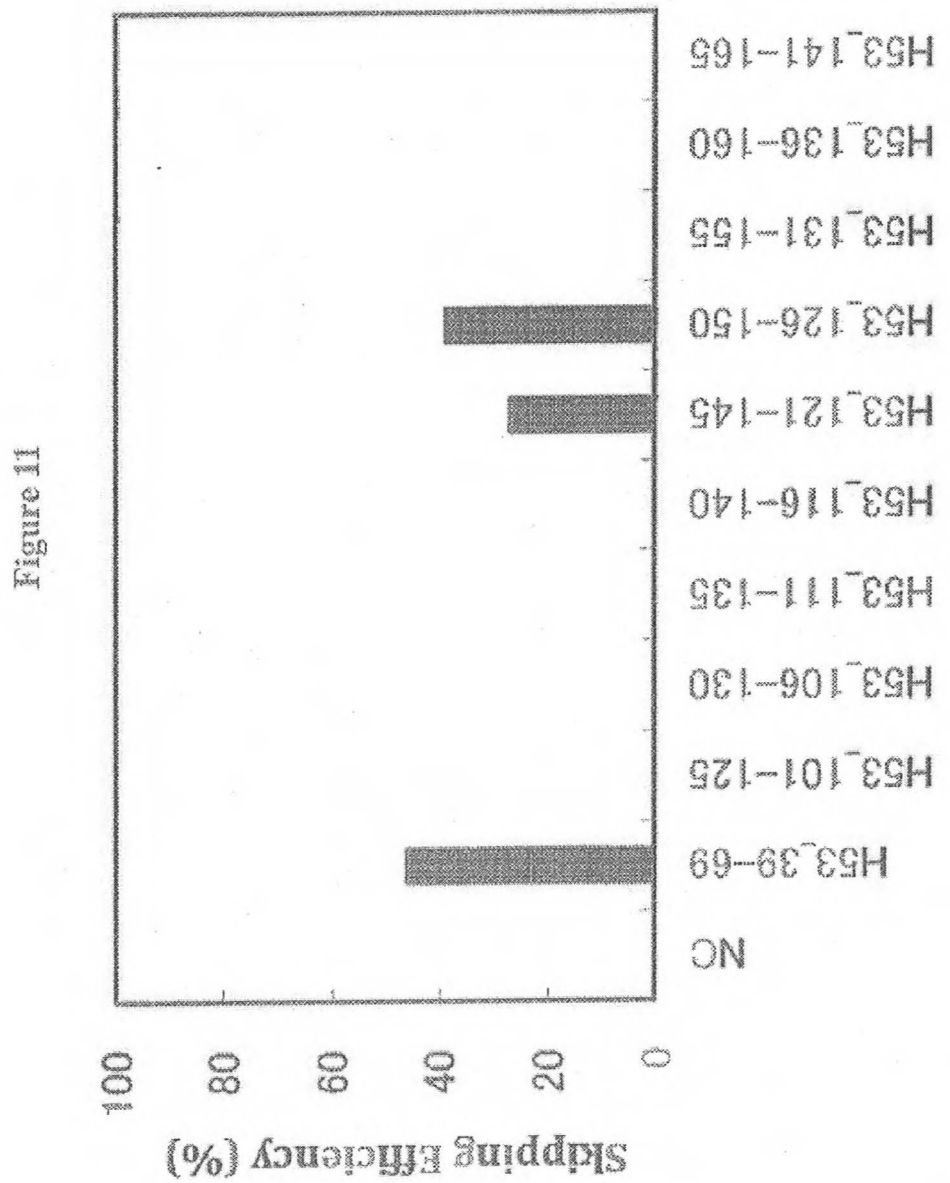

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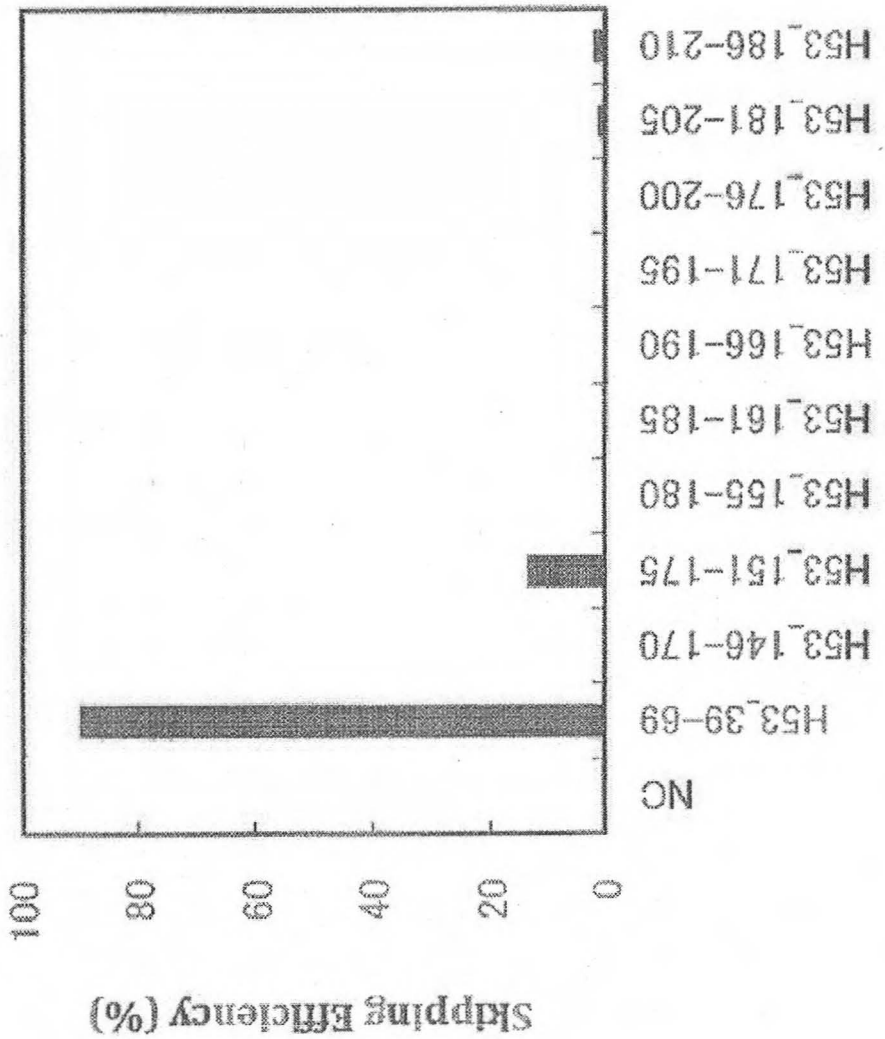

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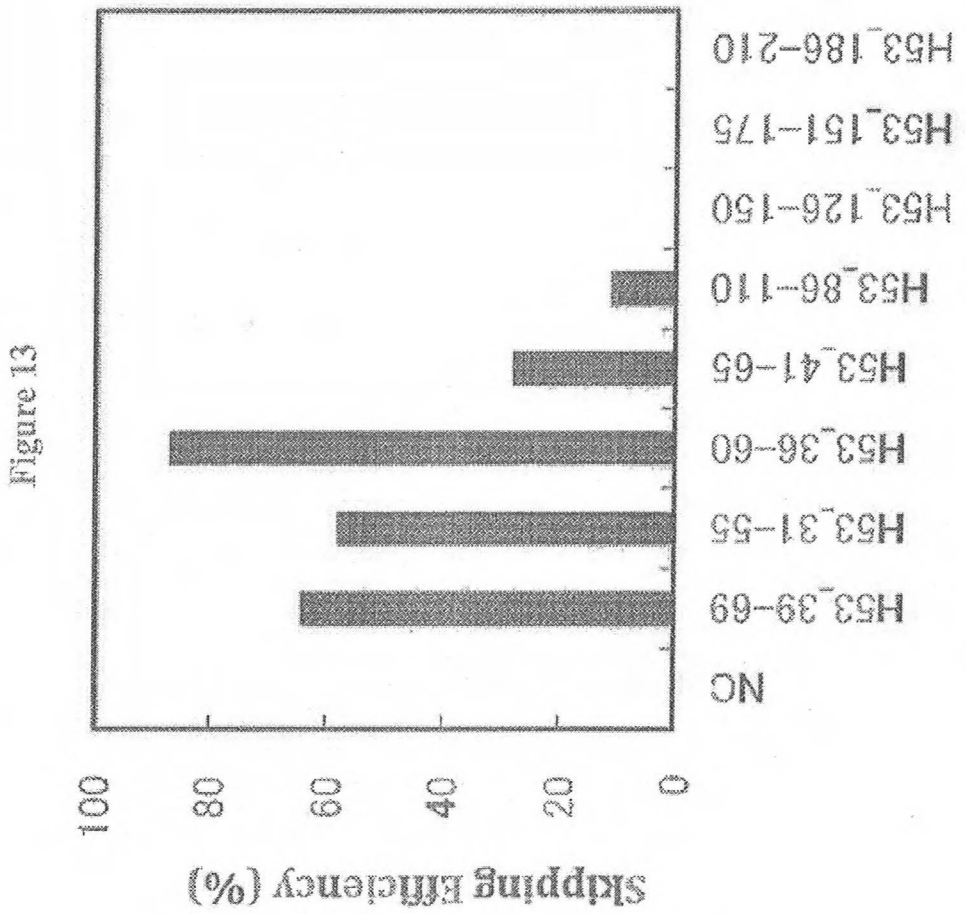

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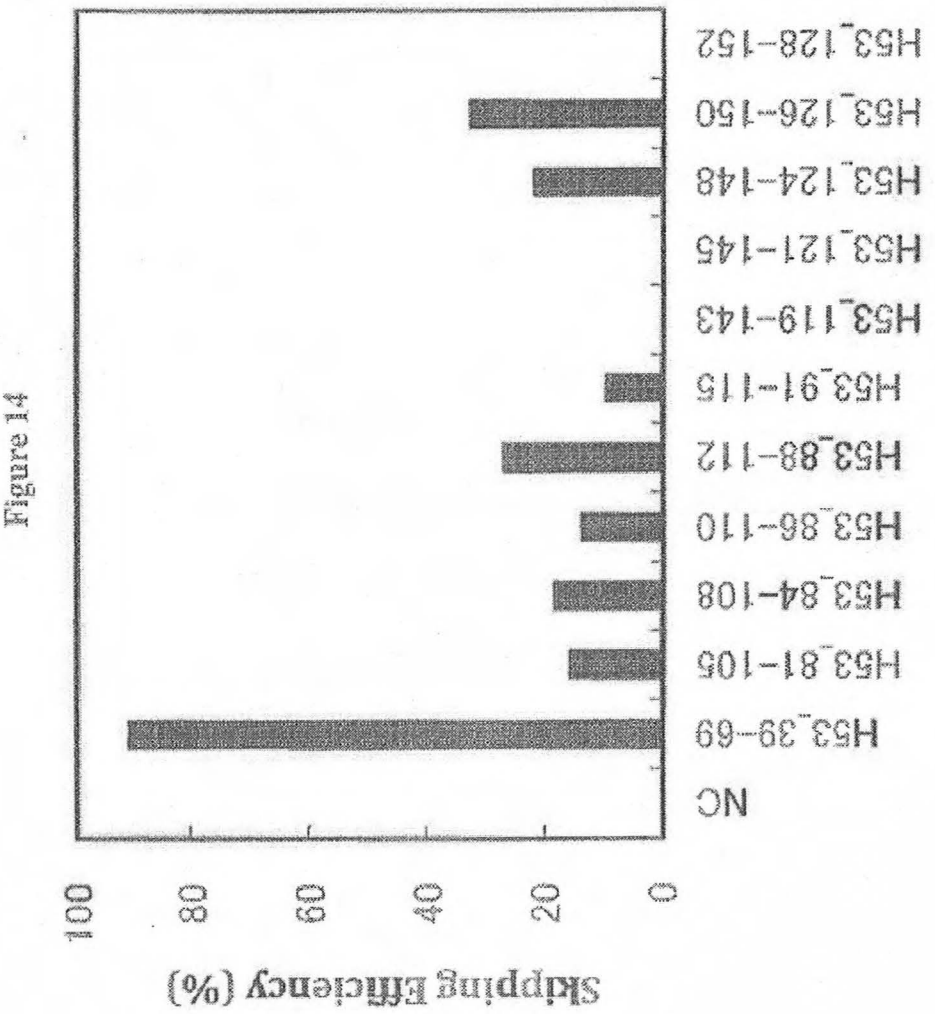

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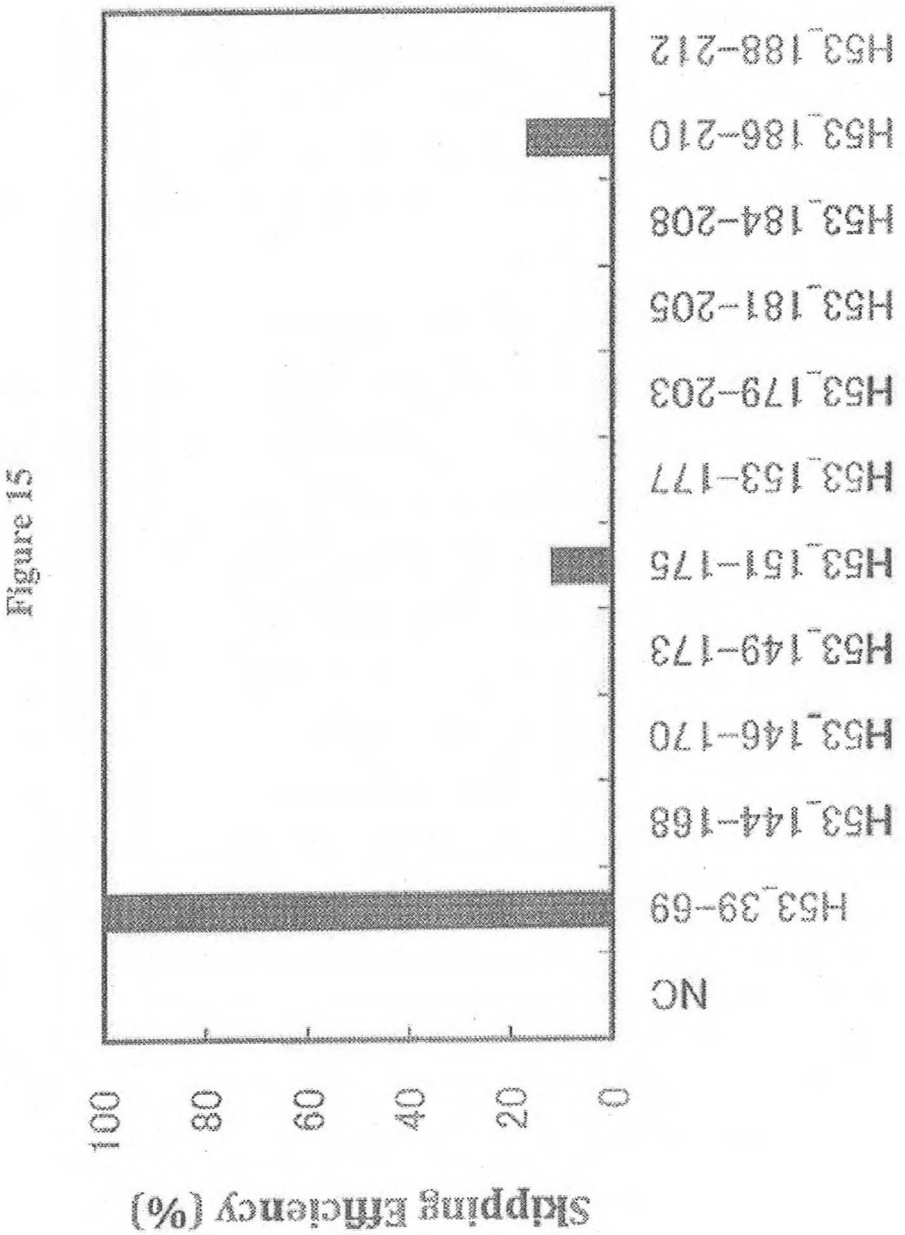


Figure 15

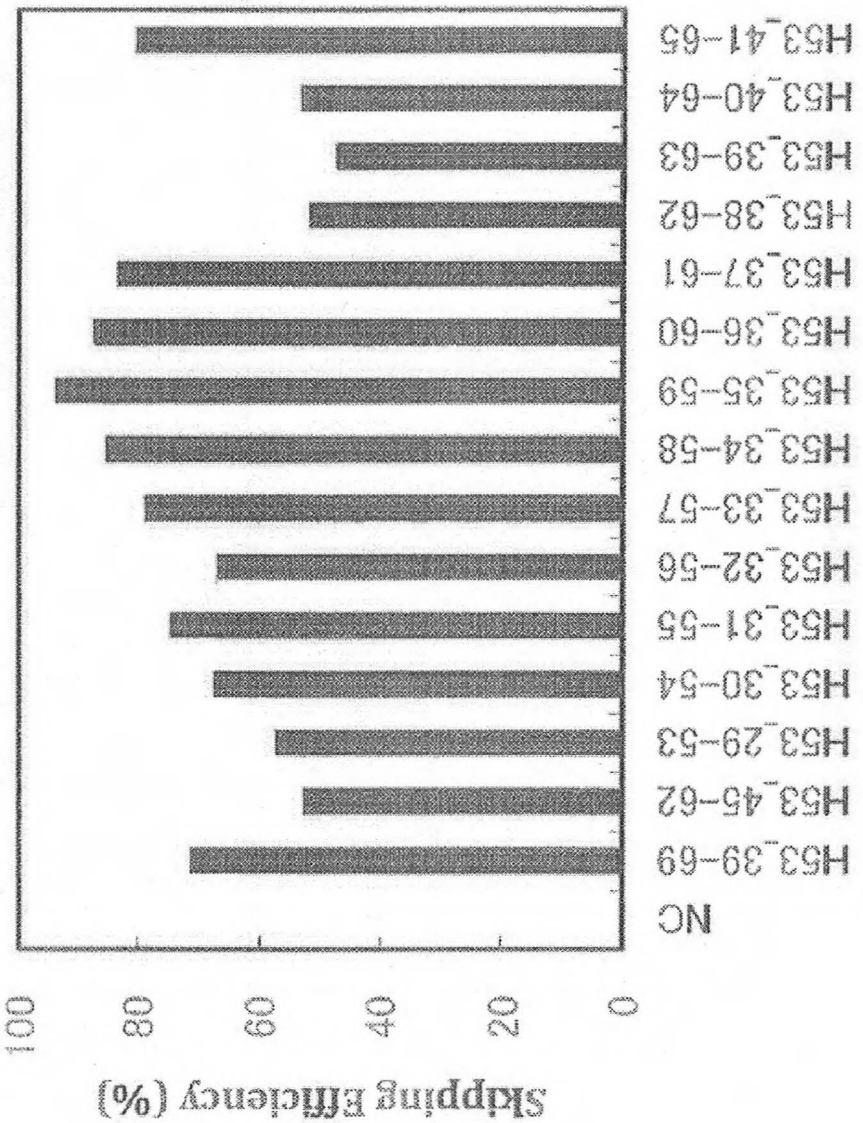


Figure 16

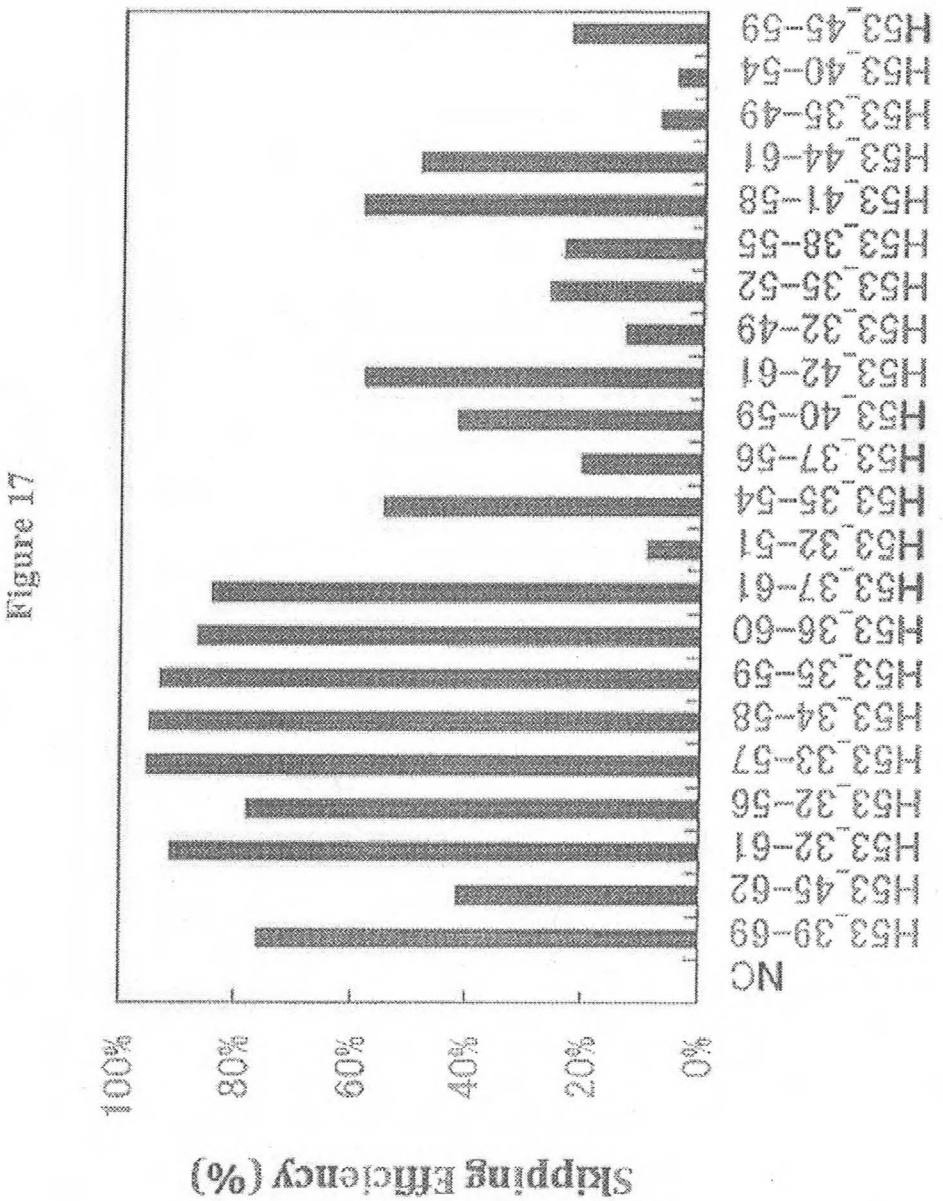


Figure 17

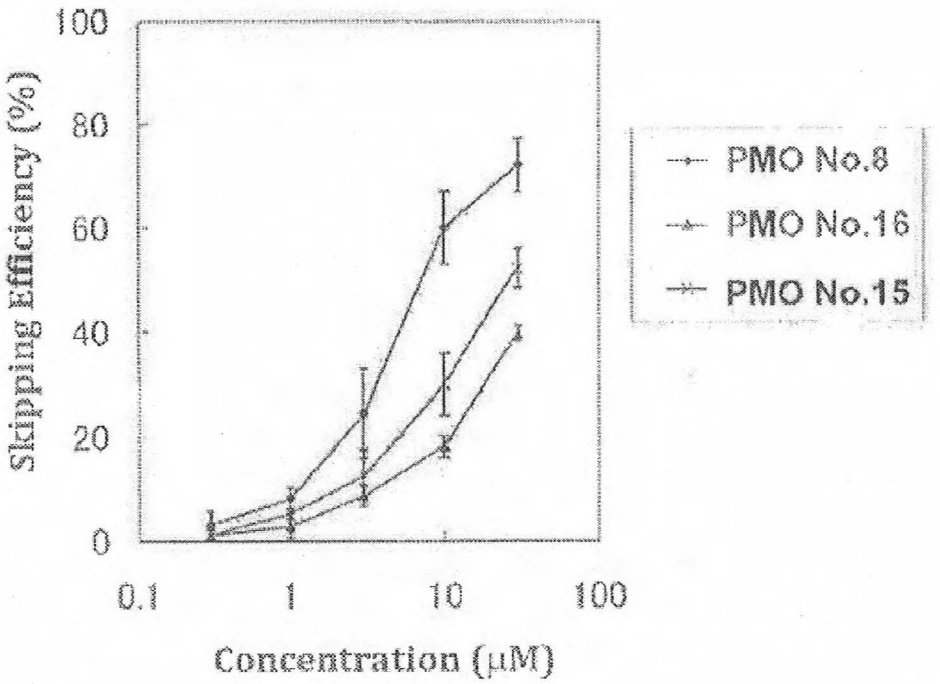


Figure 18

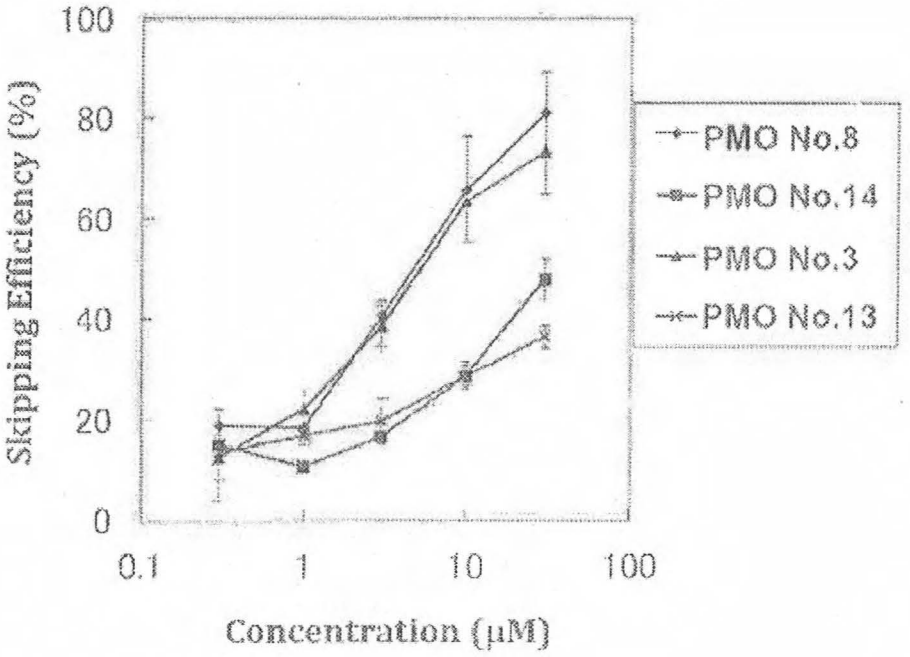


Figure 19

ANTISENSE NUCLEIC ACIDS

CROSS REFERENCE TO RELATED APPLICATIONS

This is a Continuation of copending application Ser. No. 13/819,520, filed Apr. 10, 2013, which is a PCT National Stage of PCT/JP2011/070318 filed Aug. 31, 2011, which claims priority to JP Application No. 2010-196032 filed Sep. 1, 2010.

SEQUENCE LISTING

A Sequence Listing containing SEQ ID NO: 1-123 is incorporated herein by reference.

TECHNICAL FIELD

The present invention relates to an antisense oligomer which causes skipping of exon 53 in the human dystrophin gene, and a pharmaceutical composition comprising the oligomer.

BACKGROUND ART

Duchenne muscular dystrophy (DMD) is the most frequent form of hereditary progressive muscular dystrophy that affects one in about 3,500 newborn boys. Although the motor functions are rarely different from healthy humans in infancy and childhood, muscle weakness is observed in children from around 4 to 5 years old. Then, muscle weakness progresses to the loss of ambulation by about 12 years old and death due to cardiac or respiratory insufficiency in the twenties. DMD is such a severe disorder. At present, there is no effective therapy for DMD available, and it has been strongly desired to develop a novel therapeutic agent.

DMD is known to be caused by a mutation in the dystrophin gene. The dystrophin gene is located on X chromosome and is a huge gene consisting of 2.2 million DNA nucleotide pairs. DNA is transcribed into mRNA precursors, and introns are removed by splicing to synthesize mRNA in which 79 exons are joined together. This mRNA is translated into 3,685 amino acids to produce the dystrophin protein. The dystrophin protein is associated with the maintenance of membrane stability in muscle cells and necessary to make muscle cells less fragile. The dystrophin gene from patients with DMD contains a mutation and hence, the dystrophin protein, which is functional in muscle cells, is rarely expressed. Therefore, the structure of muscle cells cannot be maintained in the body of the patients with DMD, leading to a large influx of calcium ions into muscle cells. Consequently, an inflammation-like response occurs to promote fibrosis so that muscle cells can be regenerated only with difficulty.

Becker muscular dystrophy (BMD) is also caused by a mutation in the dystrophin gene. The symptoms involve muscle weakness accompanied by atrophy of muscle but are typically mild and slow in the progress of muscle weakness, when compared to DMD. In many cases, its onset is in adulthood. Differences in clinical symptoms between DMD and BMD are considered to reside in whether the reading frame for amino acids on the translation of dystrophin mRNA into the dystrophin protein is disrupted by the mutation or not (Non-Patent Document 1). More specifically, in DMD, the presence of mutation shifts the amino acid reading frame so that the expression of functional dystrophin protein is abolished, whereas in BMD the dystrophin protein that functions, though imperfectly, is produced because the amino acid reading frame is preserved, while a part of the exons are deleted by the mutation.

Exon skipping is expected to serve as a method for treating DMD. This method involves modifying splicing to restore the amino acid reading frame of dystrophin mRNA and induce expression of the dystrophin protein having the function partially restored (Non-Patent Document 2). The amino acid sequence part, which is a target for exon skipping, will be lost. For this reason, the dystrophin protein expressed by this treatment becomes shorter than normal one but since the amino acid reading frame is maintained, the function to stabilize muscle cells is partially retained. Consequently, it is expected that exon skipping will lead DMD to the similar symptoms to that of BMD which is milder. The exon skipping approach has passed the animal tests using mice or dogs and now is currently assessed in clinical trials on human DMD patients.

The skipping of an exon can be induced by binding of antisense nucleic acids targeting either 5' or 3' splice site or both sites, or exon-internal sites. An exon will only be included in the mRNA when both splice sites thereof are recognized by the spliceosome complex. Thus, exon skipping can be induced by targeting the splice sites with antisense nucleic acids. Furthermore, the binding of an SR protein to an exonic splicing enhancer (ESE) is considered necessary for an exon to be recognized by the splicing mechanism. Accordingly, exon skipping can also be induced by targeting ESE.

Since a mutation of the dystrophin gene may vary depending on DMD patients, antisense nucleic acids need to be desined based on the site or type of respective genetic mutation. In the past, antisense nucleic acids that induce exon skipping for all 79 exons were produced by Steve Wilton, et al., University of Western Australia (Non-Patent Document 3), and the antisense nucleic acids which induce exon skipping for 39 exons were produced by Annemieke Aartsma-Rus, et al., Netherlands (Non-Patent Document 4).

It is considered that approximately 8% of all DMD patients may be treated by skipping the 53rd exon (hereinafter referred to as "exon 53"). In recent years, a plurality of research organizations reported on the studies where exon 53 in the dystrophin gene was targeted for exon skipping (Patent Documents 1 to 4; Non-Patent Document 5). However, a technique for skipping exon 53 with a high efficiency has not yet been established.

Patent Document 1: International Publication WO 2006/000057

Patent Document 2: International Publication WO 2004/048570

Patent Document 3: US 2010/0168212

Patent Document 4: International Publication WO 2010/048586

Non-Patent Document 1: Monaco A. P. et al., Genomics 1988; 2: p. 90-95

Non-Patent Document 2: Matsuo M., Brain Dev 1996; 18: p. 167-172

Non-Patent Document 3: Wilton S. D., et al., Molecular Therapy 2007; 15: p. 1288-96

Non-Patent Document 4: Annemieke Aartsma-Rus et al., (2002) Neuromuscular Disorders 12: S71-S77

Non-Patent Document 5: Linda J. Popplewell et al., (2010) Neuromuscular Disorders, vol. 20, no. 2, p. 102-10

DISCLOSURE OF THE INVENTION

Under the foregoing circumstances, antisense oligomers that strongly induce exon 53 skipping in the dystrophin gene and muscular dystrophy therapeutics comprising oligomers thereof have been desired.

As a result of detailed studies of the structure of the dystrophin gene, the present inventors have found that exon 53 skipping can be induced with a high efficiency by targeting the sequence consisting of the 32nd to the 56th nucleotides from the 5' end of exon 53 in the mRNA precursor (hereinafter referred to as "pre-mRNA") in the dystrophin gene with antisense oligomers. Based on this finding, the present inventors have accomplished the present invention.

That is, the present invention is as follows.

[1] An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences consisting of the 31st to the 53rd, the 31 st to the 54th, the 31st to the 55th, the 31st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th, or the 36th to the 58th nucleotides, from the 5' end of the 53rd exon in the human dystrophin gene.

[2] The antisense oligomer according to [1] above, which is an oligonucleotide.

[3] The antisense oligomer according to [2] above, wherein the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified.

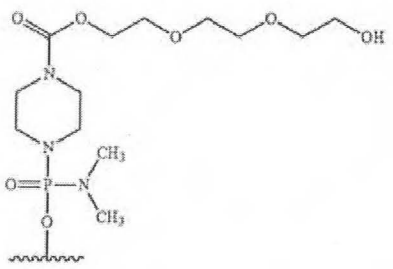
[4] The antisense oligomer according to [3] above, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of OR, R, R'OR, SiH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br and I (wherein R is an alkyl or an aryl and R' is an alkylene).

[5] The antisense oligomer according to [3] or [4] above, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond and a boranophosphate bond.

[6] The antisense oligomer according to [1] above, which is a morpholino oligomer.

[7] The antisense oligomer according to [6] above, which is a phosphorodiamidate morpholino oligomer.

[8] The antisense oligomer according to any one of [1] to [7] above, wherein the 5' end is any one of the groups of chemical formulae (1) to (3) below:



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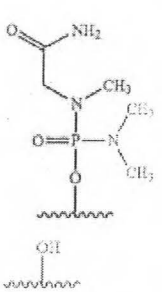


(2)

(3)

[9] The antisense oligomer according to any one of [1] to [8] above, consisting of a nucleotide sequence complementary to the sequences consisting of the 32nd to the 56th or the 36th to the 56th nucleotides from the 5' end of the 53rd exon in the human dystrophin gene.

[10] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by any one selected from the group consisting of SEQ ID NOS: 2 to 37.

[11] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by any one selected from the group consisting of SEQ ID NOS: 11, 17, 23, 29 and 35.

[12] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by SEQ ID NO: 11 or 35.

[13] A pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the antisense oligomer according to any one of [1] to [12] above, or a pharmaceutically acceptable salt or hydrate thereof.

The antisense oligomer of the present invention can induce exon 53 skipping in the human dystrophin gene with a high efficiency. In addition, the symptoms of Duchenne muscular dystrophy can be effectively alleviated by administering the pharmaceutical composition of the present invention.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cell line (RD cells).

FIG. 2 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into human normal tissue-derived fibroblasts (TIG-119 cells) to induce differentiation into muscle cells.

FIG. 3 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into human DMD patient-derived fibroblasts (5017 cells) to induce differentiation into muscle cells.

FIG. 4 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52) to induce differentiation into muscle cells.

FIG. 5 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. 6 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. 7 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52 or deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. 8 shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52) to induce differentiation into muscle cells.

FIG. 9 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 10 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 11 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 12 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 13 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 14 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 15 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 16 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 17 shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. 18 shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at the respective concentrations of the oligomers.

FIG. 19 shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at the respective concentrations of the oligomers.

BEST MODE FOR CARRYING OUT THE INVENTION

Hereinafter, the present invention is described in detail. The embodiments described below are intended to be presented by way of example merely to describe the invention but not limited only to the following embodiments. The present invention may be implemented in various ways without departing from the gist of the invention.

All of the publications, published patent applications, patents and other patent documents cited in the specification are herein incorporated by reference in their entirety. The specification hereby incorporates by reference the contents of the specification and drawings in the Japanese Patent Application (No. 2010-196032) filed Sep. 1, 2010, from which the priority was claimed.

1. Antisense Oligomer

The present invention provides the antisense oligomer (hereinafter referred to as the "oligomer of the present invention") which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences (hereinafter also referred to as "target sequences") consisting of the 31 st to the 53rd, the 31 st to the 54th, the 31 st to the 55th, the 31 st to the 56th, the 31 st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th, or the 36th to the 58th nucleotides, from the 5' end of the 53rd exon in the human dystrophin gene.

[Exon 53 in Human Dystrophin Gene]

In the present invention, the term "gene" is intended to mean a genomic gene and also include cDNA, mRNA precursor and mRNA. Preferably, the gene is mRNA precursor, i.e., pre-mRNA.

In the human genome, the human dystrophin gene locates at locus Xp21.2. The human dystrophin gene has a size of 3.0 Mbp and is the largest gene among known human genes. However, the coding regions of the human dystrophin gene are only 14 kb, distributed as 79 exons throughout the human dystrophin gene (Roberts, R G., et al., Genomics, 16: 536-538 (1993)). The pre-mRNA, which is the transcript of the human dystrophin gene, undergoes splicing to generate mature mRNA of 14 kb. The nucleotide sequence of human wild-type dystrophin gene is known (GenBank Accession No. NM_004006).

The nucleotide sequence of exon 53 in the human wild-type dystrophin gene is represented by SEQ ID NO: 1.

The oligomer of the present invention is designed to cause skipping of exon 53 in the human dystrophin gene, thereby modifying the protein encoded by DMD type of dystrophin gene into the BMD type of dystrophin protein. Accordingly, exon 53 in the dystrophin gene that is the target of exon skipping by the oligomer of the present invention includes both wild and mutant types.

Specifically, exon 53 mutants of the human dystrophin gene include the polynucleotides defined in (a) or (b) below.

(a) A polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1; and,

(b) A polynucleotide consisting of a nucleotide sequence having at least 90% identity with the nucleotide sequence of SEQ ID NO: 1.

As used herein, the term "polynucleotide" is intended to mean DNA or RNA.

As used herein, the term "polynucleotide that hybridizes under stringent conditions" refers to, for example, a polynucleotide obtained by colony hybridization, plaque hybridization, Southern hybridization or the like, using as a probe all or part of a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of, e.g., SEQ ID NO: 1. The hybridization method which may be used includes methods described in, for example, "Sambrook & Russell, Molecular Cloning: A Laboratory Manual Vol. 3, Cold Spring Harbor, Laboratory Press 2001,"

"Ausubel, Current Protocols in Molecular Biology, John Wiley & Sons 1987-1997," etc.

As used herein, the term "complementary nucleotide sequence" is not limited only to nucleotide sequences that form Watson-Crick pairs with target nucleotide sequences, but is intended to also include nucleotide sequences which form Wobble base pairs. As used herein, the term Watson-Crick pair refers to a pair of nucleobases in which hydrogen bonds are formed between adenine-thymine, adenine-uracil or guanine-cytosine, and the term Wobble base pair refers to a pair of nucleobases in which hydrogen bonds are formed between guanine-uracil, inosine-uracil, inosine-adenine or inosine-cytosine. As used herein, the term "complementary nucleotide sequence" does not only refers to a nucleotide sequence 100% complementary to the target nucleotide sequence but also refers to a complementary nucleotide sequence that may contain, for example, 1 to 3, 1 or 2, or one nucleotide non-complementary to the target nucleotide sequence.

As used herein, the term "stringent conditions" may be any of low stringent conditions, moderate stringent conditions or high stringent conditions. The term "low stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 32° C. The term "moderate stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 42° C., or 5×SSC, 1% SDS, 50 mM Tris-HCl (pH 7.5), 50% formamide at 42° C. The term "high stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 50° C. or 0.2×SSC, 0.1% SDS at 65° C. Under these conditions, polynucleotides with higher homology are expected to be obtained efficiently at higher temperatures, although multiple factors are involved in hybridization stringency including temperature, probe concentration, probe length, ionic strength, time, salt concentration and others, and those skilled in the art may appropriately select these factors to achieve similar stringency.

When commercially available kits are used for hybridization, for example, an Alkphos Direct Labeling and Detection System (GE Healthcare) may be used. In this case, according to the attached protocol, after cultivation with a labeled probe overnight, the membrane is washed with a primary wash buffer containing 0.1% (w/v) SDS at 55° C., thereby detecting hybridized polynucleotides. Alternatively, in producing a probe based on the entire or part of the nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1, hybridization can be detected with a DIG Nucleic Acid Detection Kit (Roche Diagnostics) when the probe is labeled with digoxigenin (DIG) using a commercially available reagent (e.g., a PCR Labeling Mix (Roche Diagnostics), etc.).

In addition to the polynucleotides described above, other polynucleotides that can be hybridized include polynucleotides having 90% or higher, 91% or higher, 92% or higher, 93% or higher, 94% or higher, 95% or higher, 96% or higher, 97% or higher, 98% or higher, 99% or higher, 99.1% or higher, 99.2% or higher, 99.3% or higher, 99.4% or higher, 99.5% or higher, 99.6% or higher, 99.7% or higher, 99.8% or higher or 99.9% or higher identity with the polynucleotide of SEQ ID NO: 1, as calculated by homology search software BLAST using the default parameters.

The identity between nucleotide sequences may be determined using algorithm BLAST (Basic Local Alignment Search Tool) by Karlin and Altschul (Proc. Natl. Acad. Sci. USA 872264-2268, 1990; Proc. Natl. Acad. Sci. USA 90: 5873, 1993). Programs called BLASTN and BLASTX based on the BLAST algorithm have been developed (Altschul S F, et al: J. Mol. Biol. 215: 403, 1990). When a nucleotide sequence is sequenced using BLASTN, the parameters are, for example, score=100 and wordlength=12. When BLAST and Gapped BLAST programs are used, the default parameters for each program are employed.

Examples of the nucleotide sequences complementary to the sequences consisting of the 31 st to the 53rd, the 31 st to the 54th, the 31 st to the 55th, the 31 st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th and the 36th to the 58th nucleotides, from the 5' end of exon 53.

TABLE 1

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NO:
31-53	5'-CCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 2
31-54	5'-TCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 3
31-55	5'-CTCCNGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 4
31-56	5'-GCTCCNGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 5
31-57	5'-GCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 6
31-58	5'-TGCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 7
32-53	5'-CCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 8
32-54	5'-TCCGTTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 9
32-55	5'-CTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 10
32-56	5'CCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 11
32-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 12

TABLE 1-continued

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NO:
32-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 13
33-53	5'-CCCGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 14
33-54	5'-TCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 15
33-55	5'-CTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 16
33-56	5'-CCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 17
33-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 18
33-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 19
34-53	5'-CCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 20
34-54	5'TCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 21
34-55	5'-CTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 22
34-56	5'-CCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 23
34-57	5'-GCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 24
34-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 25
35-53	5'-CCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 26
35-54	5'-TCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 27
35-55	5'-CTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 28
35-56	5'-CCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 29
35-57	5'-GCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 30
35-58	5'TGCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 31
36-53	5'-CCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 32
36-54	5'-TCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 33
36-55	5'-CTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 34
36-56	5'-CCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 35
36-57	5'-GCCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 36
36-58	5'-TGCCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 37

It is preferred that the oligomer of the present invention consists of a nucleotide sequence complementary to any one of the sequences consisting of the 32nd to the 56th, the 33rd to the 56th, the 34th to the 56th, the 35th to the 56th or the 36th to the 56th nucleotides (e.g., SEQ ID NO: 11, SEQ ID NO: 17, SEQ ID NO: 23, SEQ ID NO: 29 or SEQ ID NO: 35), from the 5' end of the 53rd exon in the human dystrophin gene.

Preferably, the oligomer of the present invention consists of a nucleotide sequence complementary to any one of the sequences consisting of the 32nd to the 56th or the 36th to the 56th nucleotides (e.g., SEQ ID NO: 11 or SEQ ID NO: 35), from the 5' end of the 53rd exon in the human dystrophin gene.

The term "cause skipping of the 53rd exon in the human dystrophin gene" is intended to mean that by binding of the oligomer of the present invention to the site corresponding to exon 53 of the transcript (e.g., pre-mRNA) of the human dystrophin gene, for example, the nucleotide sequence corresponding to the 5' end of exon 54 is spliced at the 3' side of the nucleotide sequence corresponding to the 3' end of exon 51 in DMD patients with deletion of, exon 52 when the transcript undergoes splicing, thus resulting in formation of mature mRNA which is free of codon frame shift.

Accordingly, it is not required for the oligomer of the present invention to have a nucleotide sequence 100% complementary to the target sequence, as far as it causes exon 53 skipping in the human dystrophin gene. The oligomer of the present invention may include, for example, 1 to 3, 1 or 2, or one nucleotide non-complementary to the target sequence.

Herein, the term "binding" described above is intended to mean that when the oligomer of the present invention is mixed with the transcript of human dystrophin gene, both are hybridized under physiological conditions to form a double strand nucleic acid. The term "under physiological conditions" refers to conditions set to mimic the in vivo environment in terms of pH, salt composition and tempera-

ture. The conditions are, for example, 25 to 40° C., preferably 37° C., pH 5 to 8, preferably pH 7.4 and 150 mM of sodium chloride concentration.

Whether the skipping of exon 53 in the human dystrophin gene is caused or not can be confirmed by introducing the oligomer of the present invention into a dystrophin expression cell (e.g., human rhabdomyosarcoma cells), amplifying the region surrounding exon 53 of mRNA of the human dystrophin gene from the total RNA of the dystrophin expression cell by RT-PCR and performing nested PCR or sequence analysis on the PCR amplified product.

The skipping efficiency can be determined as follows. The mRNA for the human dystrophin gene is collected from test cells; in the mRNA, the polynucleotide level "A" of the band where exon 53 is skipped and the polynucleotide level "B" of the band where exon 53 is not skipped are measured. Using these measurement values of "A" and "B," the efficiency is calculated by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A+B)\times 100$$

The oligomer of the present invention includes, for example, an oligonucleotide, morpholino oligomer or peptide nucleic acid (PNA), having a length of 18 to 28 nucleotides. The length is preferably from 21 to 25 nucleotides and morpholino oligomers are preferred.

The oligonucleotide described above (hereinafter referred to as "the oligonucleotide of the present invention") is the oligomer of the present invention composed of nucleotides as constituent units. Such nucleotides may be any of ribonucleotides, deoxyribonucleotides and modified nucleotides.

The modified nucleotide refers to one having fully or partly modified nucleobases, sugar moieties and/or phosphate-binding regions, which constitute the ribonucleotide or deoxyribonucleotide.

The nucleobase includes, for example, adenine, guanine, hypoxanthine, cytosine, thymine, uracil, and modified bases thereof. Examples of such modified nucleobases include, but not limited to, pseudouracil, 3-methyluracil, dihydrouracil, 5-alkylcytosines (e.g., 5-methylcytosine), 5-alkyluracils (e.g., 5-ethyluracil), 5-halouracils (5-bromouracil), 6-azapyrimidine, 6-alkylpyrimidines (6-methyluracil), 2-thiouracil, 4-thiouracil, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5'-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, 1-methyladenine, 1-methylhypoxanthine, 2,2-dimethylguanine, 3-methylcytosine, 2-methyladenine, 2-methylguanine, N6-methyladenine, 7-methylguanine, 5-methoxyaminomethyl-2-thiouracil, 5-methylaminomethyluracil, 5-methylcarbonylmethyluracil, 5-methyloxyuracil, 5-methyl-2-thiouracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid, 2-thiocytosine, purine, 2,6-diaminopurine, 2-aminopurine, isoguanine, indole, imidazole, xanthine, etc.

Modification of the sugar moiety may include, for example, modifications at the 2'-position of ribose and modifications of the other positions of the sugar. The modification at the 2'-position of ribose includes replacement of the 2'-OH of ribose with OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br or I, wherein R represents an alkyl or an aryl and R' represents an alkylene.

The modification for the other positions of the sugar includes, for example, replacement of O at the 4' position of ribose or deoxyribose with S, bridging between 2' and 4' positions of the sugar, e.g., LNA (locked nucleic acid) or ENA (2'-O,4'-C-ethylene-bridged nucleic acids), but is not limited thereto.

A modification of the phosphate-binding region includes, for example, a modification of replacing phosphodiester bond with phosphorothioate bond, phosphorodithioate bond, alkyl phosphonate bond, phosphoroamidate bond or boranophosphate bond (Enya et al. Bioorganic & Medicinal Chemistry, 2008, 18, 9154-9160) (cf., e.g., Japan Domestic Re-Publications of PCT Application Nos. 2006/129594 and 2006/038608).

The alkyl is preferably a straight or branched alkyl having 1 to 6 carbon atoms. Specific examples include methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, n-pentyl, isopentyl, neopentyl, tert-pentyl, n-hexyl and isohexyl. The alkyl may optionally be substituted. Examples of such substituents are a halogen, an alkoxy, cyano and nitro. The alkyl may be substituted with 1 to 3 substituents.

The cycloalkyl is preferably a cycloalkyl having 5 to 12 carbon atoms. Specific examples include cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, cyclodecyl and cyclododecyl.

The halogen includes fluorine, chlorine, bromine and iodine.

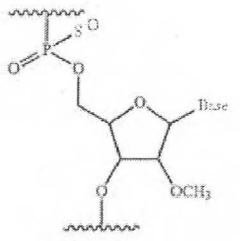
The alkoxy is a straight or branched alkoxy having 1 to 6 carbon atoms such as methoxy, ethoxy, n-propoxy, isopropoxy, n-butoxy, isobutoxy, sec-butoxy, tert-butoxy, n-pentyloxy, isopentyloxy, n-hexyloxy, isohexyloxy, etc. Among others, an alkoxy having 1 to 3 carbon atoms is preferred.

The aryl is preferably an aryl having 6 to 10 carbon atoms. Specific examples include phenyl, α-naphthyl and β-naphthyl. Among others, phenyl is preferred. The aryl may optionally be substituted. Examples of such substituents are an alkyl, a halogen, an alkoxy, cyano and nitro. The aryl may be substituted with one to three of such substituents.

The alkylene is preferably a straight or branched alkylene having 1 to 6 carbon atoms. Specific examples include methylene, ethylene, trimethylene, tetramethylene, pentamethylene, hexamethylene, 2-(ethyl)trimethylene and 1-(methyl)tetramethylene.

The acyl includes a straight or branched alkanoyl or aroyl. Examples of the alkanoyl include formyl, acetyl, 2-methylacetyl, 2,2-dimethylacetyl, propionyl, butyryl, isobutyryl, pentanoyl, 2,2-dimethylpropionyl, hexanoyl, etc. Examples of the aroyl include benzoyl, toluoyl and naphthoyl. The aroyl may optionally be substituted at substitutable positions and may be substituted with an alkyl(s).

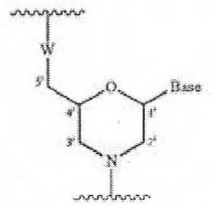
Preferably, the oligonucleotide of the present invention is the oligomer of the present invention containing a constituent unit represented by general formula below wherein the —OH group at position 2' of ribose is substituted with methoxy and the phosphate-binding region is a phosphorothioate bond:



wherein Base represents a nucleobase.

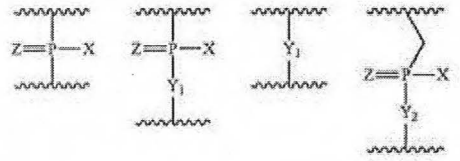
The oligonucleotide of the present invention may be easily synthesized using various automated synthesizer (e.g., AKTA oligopilot plus 10/100 (GE Healthcare)). Alternatively, the synthesis may also be entrusted to a third-party organization (e.g., Promega Inc., or Takara Co.), etc.

The morpholino oligomer of the present invention is the oligomer of the present invention comprising the constituent unit represented by general formula below:



wherein Base has the same significance as defined above, and,

W represents a group shown by any one of the following groups:



wherein

X represents $—CH_2R^1$, $—O—CH_2R^1$, $—S—CH_2R^1$, $—NR_2R^1$ or F;

R^1 represents H or an alkyl;

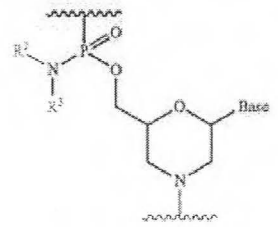
R^2 and R^3, which may be the same or different, each represents H, an alkyl, a cycloalkyl or an aryl;

Y_1 represents O, S, CH_2 or NR^1;

Y_2 represents O, S or NR^1;

Z represents O or S.

Preferably, the morpholino oligomer is an oligomer comprising a constituent unit represented by general formula below (phosphorodiamidate morpholino oligomer (hereinafter referred to as "PMO")).

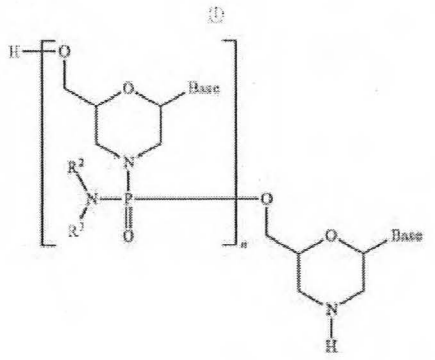


wherein Base, R^2 and R^3 have the same significance as defined above.

The morpholino oligomer may be produced in accordance with, e.g., WO 1991/009033 or WO 2009/064471. In particular, PMO can be produced by the procedure described in WO 2009/064471 or produced by the process shown below.

[Method for Producing PMO]

An embodiment of PMO is, for example, the compound represented by general formula (I) below (hereinafter PMO (I)).



wherein

Base, R^2 and R^3 have the same significance as defined above; and,

n is a given integer of 1 to 99, preferably a given integer of 18 to 28.

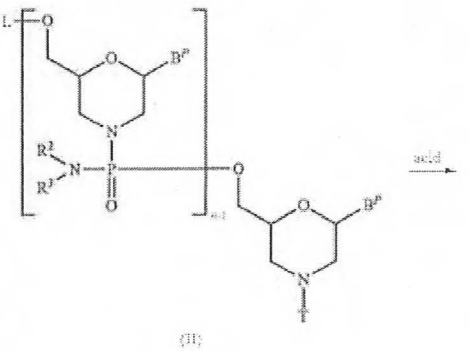
PMO (I) can be produced in accordance with a known method, for example, can be produced by performing the procedures in the following steps.

The compounds and reagents used in the steps below are not particularly limited so long as they are commonly used to prepare PMO.

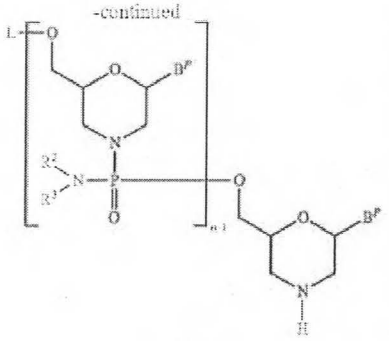
Also, the following steps can all be carried out by the liquid phase method or the solid phase method (using manuals or commercially available solid phase automated synthesizers). In producing PMO by the solid phase method, it is desired to use automated synthesizers in view of simple operation procedures and accurate synthesis.

(1) Step A:

The compound represented by general formula (II) below (hereinafter referred to as Compound (II)) is reacted with an acid to prepare the compound represented by general formula (III) below (hereinafter referred to as Compound (III)):



-continued



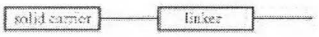
(III)

wherein n, R^2 and R^3 have the same significance as defined above;

each B^P independently represents a nucleobase which may optionally be protected;

T represents trityl, monomethoxytrityl or dimethoxytrityl; and,

L represents hydrogen, an acyl or a group represented by general formula (IV) below (hereinafter referred to as group (IV)).



(IV)

The "nucleobase" for B^P includes the same "nucleobase" as in Base, provided that the amino or hydroxy group in the nucleobase shown by B^P may be protected.

Such protective group for amino is not particularly limited so long as it is used as a protective group for nucleic acids. Specific examples include benzoyl, 4-methoxybenzoyl, acetyl, propionyl, butyryl, isobutyryl, phenylacetyl, phenoxyacetyl, 4-tert-butylphenoxyacetyl, 4-isopropylphenoxyacetyl and (dimethylamino)methylene. Specific examples of the protective group for the hydroxy group include 2-cyanoethyl, 4-nitrophenethyl, phenylsulfonylethyl, methylsulfonylethyl and trimethylsilylethyl, and phenyl, which may be substituted by 1 to 5 electron-withdrawing group at optional substitutable positions, diphenylcarbamoyl, dimethylcarbamoyl, diethylcarbamoyl, methylphenylcarbamoyl, 1-pyrolidinylcarbamoyl, morpholinocarbamoyl, 4-(tert-butylcarboxy)benzyl, 4-[(dimethylamino)carboxy]benzyl and 4-(phenylcarboxy)benzyl, (cf., e.g., WO 2009/064471).

The "solid carrier" is not particularly limited so long as it is a carrier usable for the solid phase reaction of nucleic acids. It is desired for the solid carrier to have the following properties: e.g., (i) it is sparingly soluble in reagents that can be used for the synthesis of morpholino nucleic acid derivatives (e.g., dichloromethane, acetonitrile, tetrazole, N-methylimidazole, pyridine, acetic anhydride, lutidine, trifluoroacetic acid); (ii) it is chemically stable to the reagents usable for the synthesis of morpholino nucleic acid derivatives; (iii) it can be chemically modified; (iv) it can be charged with desired morpholino nucleic acid derivatives; (v) it has a strength sufficient to withstand high pressure through treatments; and (vi) it has a uniform particle diameter range and distribution. Specifically, swellable polystyrene (e.g., aminomethyl polystyrene resin 1% dibenzylbenzene crosslinked (200-400 mesh) (2.4-3.0 mmol/g) (manufactured by Tokyo Chemical Industry), Aminomethylated Polystyrene Resin.HCl [dibenzylbenzene 1%, 100-200 mesh] (manufactured by Peptide Institute, Inc.)), non-swellable polystyrene (e.g., Primer Support (manufactured by GE Healthcare)), PEG chain-attached polystyrene (e.g., NH_2—PEG resin (manufactured by Watanabe Chemical Co.), TentaGel resin), controlled pore glass (controlled pore glass; CPG) (manufactured by, e.g., CPG), oxalyl-controlled pore glass (cf., e.g., Alul et al., Nucleic Acids Research, Vol. 19, 1527 (1991)), TentaGel support-aminopolyethylene glycol-derivatized support (e.g., Wright et al., cf., Tetrahedron Letters, Vol. 34, 3373 (1993)), and a copolymer of Poros-polystyrene/divinylbenzene.

A "linker" which can be used is a known linker generally used to connect nucleic acids or morpholino nucleic acid derivatives. Examples include 3-aminopropyl, succinyl, 2,2'-diethanolsulfonyl and a long chain alkyl amino (LCAA).

This step can be performed by reacting Compound (II) with an acid.

The "acid" which can be used in this step includes, for example, trifluoroacetic acid, dichloroacetic acid and trichloroacetic acid. The acid used is appropriately in a range of, for example, 0.1 mol equivalent to 1000 mol equivalents based on 1 mol of Compound (II), preferably in a range of 1 mol equivalent to 100 mol equivalents based on 1 mol of Compound (II).

An organic amine can be used in combination with the acid described above. The organic amine is not particularly limited and includes, for example, triethylamine. The amount of the organic amine used is appropriately in a range of, e.g., 0.01 mol equivalent to 10 mol equivalents, and preferably in a range of 0.1 mol equivalent to 2 mol equivalents, based on 1 mol of the acid.

When a salt or mixture of the acid and the organic amine is used in this step, the salt or mixture includes, for example, a salt or mixture of trifluoroacetic acid and triethylamine, and more specifically, a mixture of 1 equivalent of triethylamine and 2 equivalents of trifluoroacetic acid.

The acid which can be used in this step may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, or a mixture thereof.

The reaction temperature in the reaction described above is preferably in a range of, e.g., 10° C. to 50° C., more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C.

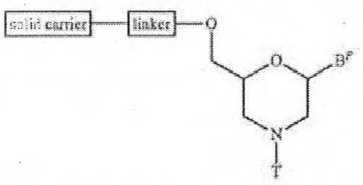
The reaction time may vary depending upon kind of the acid used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

After completion of this step, a base may be added, if necessary, to neutralize the acid remained in the system. The "base" is not particularly limited and includes, for example, diisopropylamine. The base may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% (v/v) to 30% (v/v).

The solvent used in this step is not particularly limited so long as it is inert to the reaction, and includes dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, and a mixture thereof. The reaction temperature is preferably in a range of, e.g., 10° C. to 50° C., more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C.

The reaction time may vary depending upon kind of the base used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

In Compound (II), the compound of general formula (IIa) below (hereinafter Compound (IIa)), wherein n is 1 and L is a group (IV), can be produced by the following procedure.

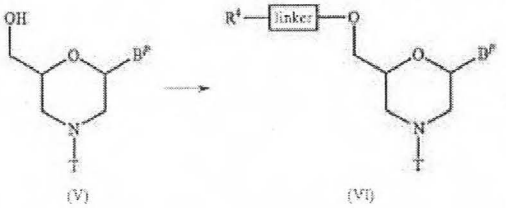


(IIa)

wherein B^P, T, linker and solid carrier have the same significance as defined above.

Step 1

The compound represented by general formula (V) below is reacted with an acylating agent to prepare the compound represented by general formula (VI) below (hereinafter referred to as Compound (VI)).



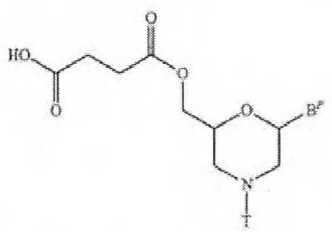
(V) (VI)

wherein B^P, T and linker have the same significance as defined above; and,

R^4 represents hydroxy, a halogen or amino.

This step can be carried out by known procedures for introducing linkers, using Compound (V) as the starting material.

In particular, the compound represented by general formula (VIa) below can be produced by performing the method known as esterification, using Compound (V) and succinic anhydride.

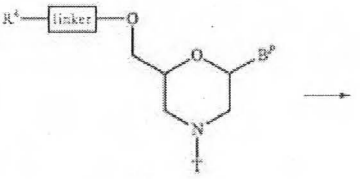


(VIa)

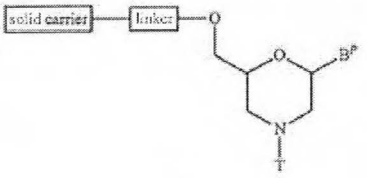
wherein B^P and T have the same significance as defined above.

Step 2

Compound (VI) is reacted with a solid career by a condensing agent to prepare Compound (IIa).



(VI)

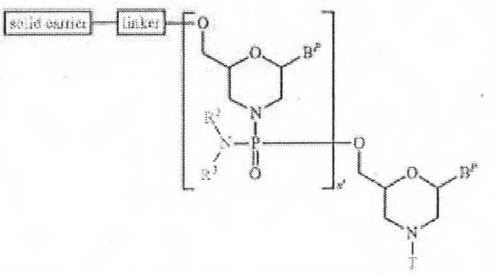


(IIa)

wherein B^P, R^4, T, linker and solid carrier have the same significance as defined above.

This step can be performed using Compound (VI) and a solid carrier in accordance with a process known as condensation reaction.

In Compound (II), the compound represented by general formula (IIa2) below wherein n is 2 to 99 and L is a group represented by general formula (IV) can be produced by using Compound (IIa) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.



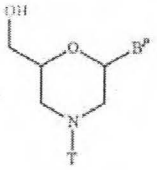
(IIa2)

wherein B^P, R^2, R^3, T, linker and solid carrier have the same significance as defined above; and,

n' represents 1 to 98.

In Compound (II), the compound of general formula (IIb) below wherein n is 1 and L is hydrogen can be produced by the procedure described in, e.g., WO 1991/009033.

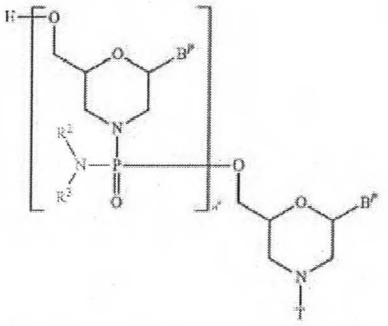
(IIb)



wherein B^P and T have the same significance as defined above.

In Compound (II), the compound represented by general formula (IIb2) below wherein n is 2 to 99 and L is hydrogen can be produced by using Compound (IIb) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

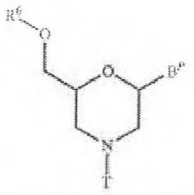
(IIb2)



wherein B^P, n', R^2, R^3 and T have the same significance as defined above.

In Compound (II), the compound represented by general formula (IIc) below wherein n is 1 and L is an acyl can be produced by performing the procedure known as acylation reaction, using Compound (IIb).

(IIc)

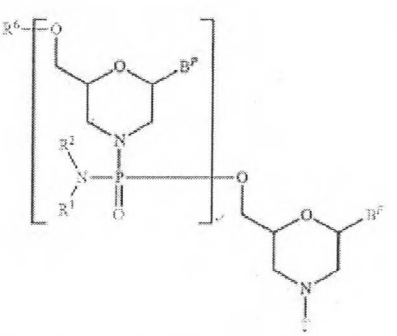


wherein B^P and T have the same significance as defined above; and,

R^5 represents an acyl.

In Compound (II), the compound represented by general formula (IIc2) below wherein n is 2 to 99 and L is an acyl can be produced by using Compound (IIc) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

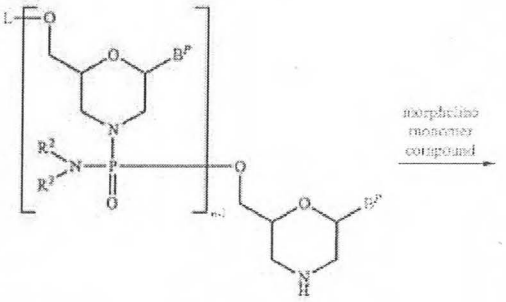
(IIc2)



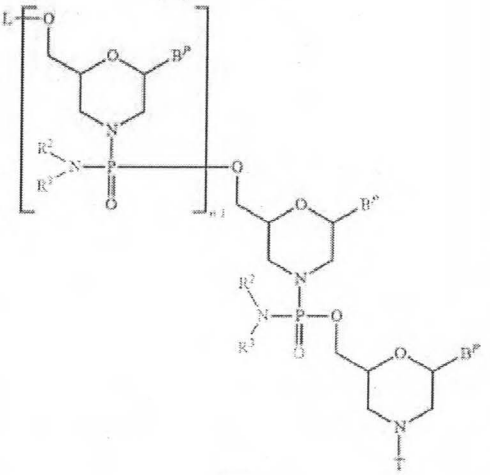
wherein B^P, n', R^2, R^3, R^5 and T have the same significance as defined above.

(2) Step B

Compound (III) is reacted with a morpholino monomer compound in the presence of a base to prepare the compound represented by general formula (VII) below (hereinafter referred to as Compound (VII)):



(III)



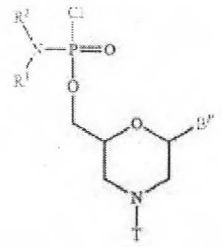
(VII)

wherein B^P, L, n, R^2, R^3 and T have the same significance as defined above.

This step can be performed by reacting Compound (III) with the morpholino monomer compound in the presence of a base.

The morpholino monomer compound includes, for example, compounds represented by general formula (VIII) below:

(VIII)



wherein B^P, R^2, R^3 and T have the same significance as defined above.

The "base" which can be used in this step includes, for example, diisopropylamine, triethylamine and N-ethylmorpholine. The amount of the base used is appropriately in a range of 1 mol equivalent to 1000 mol equivalents based on 1 mol of Compound (III), preferably, 10 mol equivalents to 100 mol equivalents based on 1 mol of Compound (III).

The morpholino monomer compound and base which can be used in this step may also be used as a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, N,N-dimethylimidazolidone, N-methylpiperidone, DMF, dichloromethane, acetonitrile, tetrahydrofuran, or a mixture thereof.

The reaction temperature is preferably in a range of, e.g., 0° C. to 100° C., and more preferably, in a range of 10° C. to 50° C.

The reaction time may vary depending upon kind of the base used and reaction temperature, and is appropriately in a range of 1 minute to 48 hours in general, and preferably in a range of 30 minutes to 24 hours.

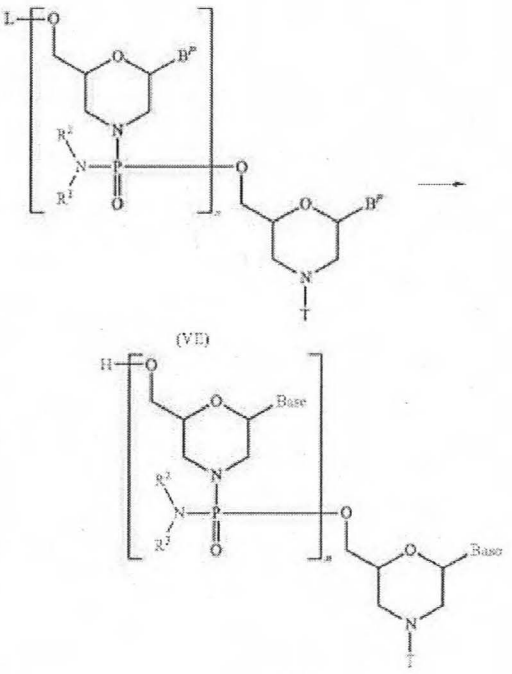
Furthermore, after completion of this step, an acylating agent can be added, if necessary. The "acylating agent" includes, for example, acetic anhydride, acetyl chloride and phenoxyacetic anhydride. The acylating agent may also be used as a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, dichloromethane, acetonitrile, an alcohol(s) (ethanol, isopropanol, trifluoroethanol, etc.), water, or a mixture thereof.

If necessary, a base such as pyridine, lutidine, collidine, triethylamine, diisopropylethylamine, N-ethylmorpholine, etc. may also be used in combination with the acylating agent. The amount of the acylating agent is appropriately in a range of 0.1 mol equivalent to 10000 mol equivalents, and preferably in a range of 1 mol equivalent to 1000 mol equivalents. The amount of the base is appropriately in a range of, e.g., 0.1 mol equivalent to 100 mol equivalents, and preferably in a range of 1 mol equivalent to 10 mol equivalents, based on 1 mol of the acylating agent.

The reaction temperature in this reaction is preferably in a range of 10° C. to 50° C., more preferably, in a range of 10° C. to 50° C., much more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C. The reaction time may vary depending upon kind of the acylating agent used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

(3) Step C:

In Compound (VII) produced in Step B, the protective group is removed using a deprotecting agent to prepare the compound represented by general formula (IX).



(IX)

wherein Base, B^P, n, R^2, R^3 and T have the same significance as defined above.

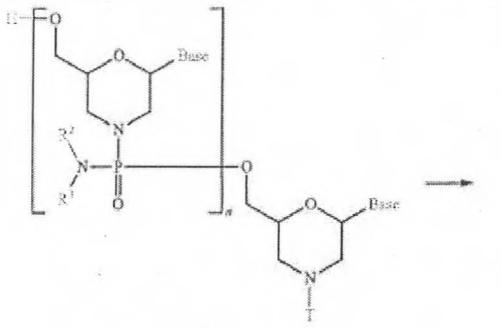
This step can be performed by reacting Compound (VII) with a deprotecting agent.

The "deprotecting agent" includes, e.g., conc. ammonia water and methylamine. The "deprotecting agent" used in this step may also be used as a dilution with, e.g., water, methanol, ethanol, isopropyl alcohol, acetonitrile, tetrahydrofuran, DMF, N,N-dimethylimidazolidone, N-methylpiperidone, or a mixture of these solvents. Among others, ethanol is preferred. The amount of the deprotecting agent used is appropriately in a range of, e.g., 1 mol equivalent to 100000 mol equivalents, and preferably in a range of 10 mol equivalents to 1000 mol equivalents, based on 1 mol of Compound (VII).

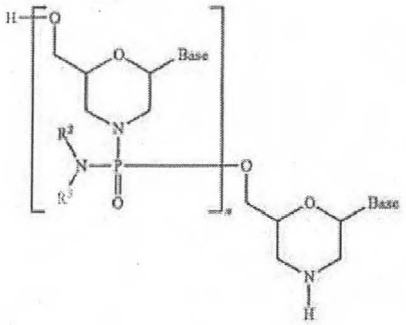
The reaction temperature is appropriately in a range of 15° C. to 75° C., preferably, in a range of 40° C. to 70° C., and more preferably, in a range of 50° C. to 60° C. The reaction time for deprotection may vary depending upon kind of Compound (VII), reaction temperature, etc., and is appropriately in a range of 10 minutes to 30 hours, preferably 30 minutes to 24 hours, and more preferably in a range of 5 hours to 20 hours.

(4) Step D:

PMO (I) is produced by reacting Compound (IX) produced in step C with an acid:



(IX)



(I)

wherein Base, n, R^2, R^3 and T have the same significance as defined above. This step can be performed by adding an acid to Compound (IX).

The "acid" which can be used in this step includes, for example, trichloroacetic acid, dichloroacetic acid, acetic acid, phosphoric acid, hydrochloric acid, etc. The acid used is appropriately used to allow the solution to have a pH range of 0.1 to 4.0, and more preferably, in a range of pH 1.0 to 3.0. The solvent is not particularly limited so long as it is inert to the reaction, and includes, for example, acetonitrile, water, or a mixture of these solvents thereof.

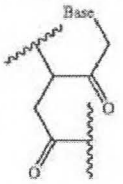
The reaction temperature is appropriately in a range of 10° C. to 50° C., preferably, in a range of 20° C. to 40° C., and more preferably, in a range of 25° C. to 35° C. The reaction time for deprotection may vary depending upon kind of Compound (IX), reaction temperature, etc., and is appropriately in a range of 0.1 minute to 5 hours, preferably 1 minute to 1 hour, and more preferably in a range of 1 minute to 30 minutes.

PMO (I) can be obtained by subjecting the reaction mixture obtained in this step to conventional means of separation and purification such as extraction, concentration, neutralization, filtration, centrifugal separation, recrystallization, reversed phase column chromatography C_8 to C_{18}, cation exchange column chromatography, anion exchange column chromatography, gel filtration column chromatography, high performance liquid chromatography, dialysis, ultrafiltration, etc., alone or in combination thereof. Thus, the desired PMO (I) can be isolated and purified (cf., e.g., WO 1991/09033).

In purification of PMO (I) using reversed phase chromatography, e.g., a solution mixture of 20 mM triethylamine/acetate buffer and acetonitrile can be used as an elution solvent.

In purification of PMO (I) using ion exchange chromatography, e.g., a solution mixture of 1 M saline solution and 10 mM sodium hydroxide aqueous solution can be used as an elution solvent.

A peptide nucleic acid is the oligomer of the present invention having a group represented by the following general formula as the constituent unit:



wherein Base has the same significance as defined above.

Peptide nucleic acids can be prepared by referring to, e.g., the following literatures.

1) P. E. Nielsen, M. Egholm, R. H. Berg, O. Buchardt, Science, 254, 1497 (1991)

2) M. Egholm, O. Buchardt, P. E. Nielsen, R. H. Berg, Jacs., 114, 1895 (1992)

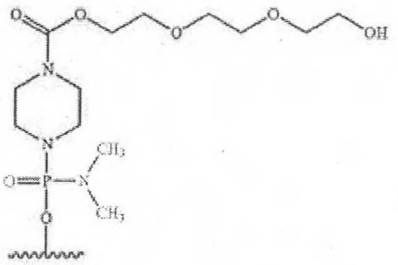
3) K. L. Dueholmn, M. Egholm, C. Behrens, L. Christensen, H. F. Hansen, T. Vulpius, K. H. Petersen, R. H. Berg, P. E. Nielsen, O. Buchardt, J. Org. Chem., 59, 5767 (1994)

4) L. Christensen, R. Fitzpatrick, B. Gildea, K. H. Petersen, H. F. Hansen, T. Koch, M. Egholm, O. Buchardt, P. E. Nielsen, J. Coull, R. H. Berg, J. Pept. Sci., 1, 175 (1995)

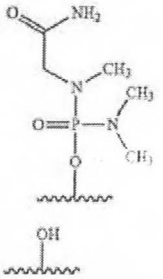
5) T. Koch, H. F. Hansen, P. Andersen, T. Larsen, H. G. Batz, K. Otteson, H. Orum, J. Pept. Res., 49, 80 (1997)

In the oligomer of the present invention, the 5' end may be any of chemical structures (1) to (3) below, and preferably is (3)-OH.

(1)



(2)



(3)

Hereinafter, the groups shown by (1), (2) and (3) above are referred to as "Group (1)," "Group (2)" and "Group (3)," respectively.

2. Pharmaceutical Composition

The oligomer of the present invention causes exon 53 skipping with a higher efficiency as compared to the prior art

antisense oligomers. It is thus expected that conditions of muscular dystrophy can be relieved with high efficience by administering the pharmaceutical composition comprising the oligomer of the present invention to DMD patients. For example, when the pharmaceutical composition comprising the oligomer of the present invention is used, the same therapeutic effects can be achieved even in a smaller dose than that of the oligomers of the prior art. Accordingly, side effects can be alleviated and such is economical.

In another embodiment, the present invention provides the pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the oligomer of the present invention, a pharmaceutically acceptable salt or hydrate thereof (hereinafter referred to as "the composition of the present invention").

Examples of the pharmaceutically acceptable salt of the oligomer of the present invention contained in the composition of the present invention are alkali metal salts such as salts of sodium, potassium and lithium; alkaline earth metal salts such as salts of calcium and magnesium; metal salts such as salts of aluminum, iron, zinc, copper, nickel, cobalt, etc.; ammonium salts; organic amine salts such as salts of t-octylamine, dibenzylamine, morpholine, glucosamine, phenylglycine alkyl ester, ethylenediamine, N-methylglucamine, guanidine, diethylamine, triethylamine, dicyclohexylamine, N,N-dibenzylethylenediamine, chloroprocaine, procaine, diethanolamine, N-benzylphenethylamine, piperazine, tetramethylammonium, tris(hydroxymethyl)aminomethane; hydrohalide salts such as salts of hydrofluorates, hydrochlorides, hydrobromides and hydroiodides; inorganic acid salts such as nitrates, perchlorates, sulfates, phosphates, etc.; lower alkane sulfonates such as methanesulfonates, trifluoromethanesulfonates and ethanesulfonates; arylsulfonates such as benzenesulfonates and p-toluenesulfonates; organic acid salts such as acetates, malates, fumarates, succinates, citrates, tartarates, oxalates, maleates, etc.; and, amino acid salts such as salts of glycine, lysine, arginine, ornithine, glutamic acid and aspartic acid. These salts may be produced by known methods. Alternatively, the oligomer of the present invention contained in the composition of the present invention may be in the form of a hydrate thereof.

Administration route for the composition of the present invention is not particularly limited so long as it is pharmaceutically acceptable route for administration, and can be chosen depending upon method of treatment. In view of easiness in delivery to muscle tissues, preferred are intravenous administration, intraarterial administration, intramuscular administration, subcutaneous administration, oral administration, tissue administration, transdermal administration, etc. Also, dosage forms which are available for the composition of the present invention are not particularly limited, and include, for example, various injections, oral agents, drips, inhalations, ointments, lotions, etc.

In administration of the oligomer of the present invention to patients with muscular dystrophy, the composition of the present invention preferably contains a carrier to promote delivery of the oligomer to muscle tissues. Such a carrier is not particularly limited as far as it is pharmaceutically acceptable, and examples include cationic carriers such as cationic liposomes, cationic polymers, etc., or carriers using viral envelope. The cationic liposomes are, for example, liposomes composed of 2-O-(2-diethylaminoethyl)carabamoyl-1,3-O-dioleoylglycerol and phospholipids as the essential constituents (hereinafter referred to as "liposome A"), Oligofectamine (registered trademark) (manufactured by Invitrogen Corp.), Lipofectin (registered trademark) (manufactured by Invitrogen Corp.), Lipofectamine (registered trademark) (manufactured by Invitrogen Corp.), Lipofectamine 2000 (registered trademark) (manufactured by Invitrogen Corp.), DMRIE-C (registered trademark) (manufactured by Invitrogen Corp.), GeneSilencer (registered trademark) (manufactured by Gene Therapy Systems), TransMessenger (registered trademark) (manufactured by QIAGEN, Inc.), TransIT TKO (registered trademark) (manufactured by Mirus) and Nucleofector II (Lonza). Among others, liposome A is preferred. Examples of cationic polymers are JetSI (registered trademark) (manufactured by Qbiogene, Inc.) and Jet-PEI (registered trademark) (polyethylenimine, manufactured by Qbiogene, Inc.). An example of carriers using viral envelop is GenomeOne (registered trademark) (HVJ-E liposome, manufactured by Ishihara Sangyo). Alternatively, the medical devices described in Japanese Patent No. 2924179 and the cationic carriers described in Japanese Domestic Re-Publication PCT Nos. 2006/129594 and 2008/096690 may be used as well.

A concentration of the oligomer of the present invention contained in the composition of the present invention may vary depending on kind of the carrier, etc., and is appropriately in a range of 0.1 nM to 100 μM, preferably in a range of 1 nM to 10 μM, and more preferably in a range of 10 nM to 1 μM. A weight ratio of the oligomer of the present invention contained in the composition of the present invention and the carrier (carrier/oligomer of the present invention) may vary depending on property of the oligomer, type of the carrier, etc., and is appropriately in a range of 0.1 to 100, preferably in a range of 1 to 50, and more preferably in a range of 10 to 20.

In addition to the oligomer of the present invention and the carrier described above, pharmaceutically acceptable additives may also be optionally formulated in the composition of the present invention. Examples of such additives are emulsification aids (e.g., fatty acids having 6 to 22 carbon atoms and their pharmaceutically acceptable salts, albumin and dextran), stabilizers (e.g., cholesterol and phosphatidic acid), isotonizing agents (e.g., sodium chloride, glucose, maltose, lactose, sucrose, trehalose), and pH controlling agents (e.g., hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid, sodium hydroxide, potassium hydroxide and triethanolamine). One or more of these additives can be used. The content of the additive in the composition of the present invention is appropriately 90 wt % or less, preferably 70 wt % or less and more preferably, 50 wt % or less.

The composition of the present invention can be prepared by adding the oligomer of the present invention to a carrier dispersion and adequately stirring the mixture. Additives may be added at an appropriate step either before or after addition of the oligomer of the present invention. An aqueous solvent that can be used in adding the oligomer of the present invention is not particularly limited as far as it is pharmaceutically acceptable, and examples are injectable water or injectable distilled water, electrolyte fluid such as physiological saline, etc., and sugar fluid such as glucose fluid, maltose fluid, etc. A person skilled in the art can appropriately choose conditions for pH and temperature for such matter.

The composition of the present invention may be prepared into, e.g., a liquid form and its lyophilized preparation. The lyophilized preparation can be prepared by lyophilizing the composition of the present invention in a liquid form in a conventional manner. The lyophilization can be performed, for example, by appropriately sterilizing the composition of the present invention in a liquid form, dispensing an aliquot into a vial container, performing preliminary freezing for 2 hours at conditions of about −40 to −20° C., performing a primary drying at 0 to 10° C. under reduced pressure, and then performing a secondary drying at about 15 to 25° C. under reduced pressure. In general, the lyophilized prepa-

ration of the composition of the present invention can be obtained by replacing the content of the vial with nitrogen gas and capping.

The lyophilized preparation of the composition of the present invention can be used in general upon reconstitution by adding an optional suitable solution (reconstitution liquid) and redissolving the preparation. Such a reconstitution liquid includes injectable water, physiological saline and other infusion fluids. A volume of the reconstitution liquid may vary depending on the intended use, etc., is not particularly limited, and is suitably 0.5 to 2-fold greater than the volume prior to lyophilization or no more than 500 mL.

It is desired to control a dose of the composition of the present invention to be administered, by taking the following factors into account: the type and dosage form of the oligomer of the present invention contained; patients' conditions including age, body weight, etc.; administration route; and the characteristics and extent of the disease. A daily dose calculated as the amount of the oligomer of the present invention is generally in a range of 0.1 mg to 10 g/human, and preferably 1 mg to 1 g/human. This numerical range may vary occasionally depending on type of the target disease, administration route and target molecule. Therefore, a dose lower than the range may be sufficient in some occasion and conversely, a dose higher than the range may be required occasionally. The composition can be administered from once to several times daily or at intervals from one day to several days.

In still another embodiment of the composition of the present invention, there is provided a pharmaceutical composition comprising a vector capable of expressing the oligonucleotide of the present invention and the carrier described above. Such an expression vector may be a vector capable of expressing a plurality of the oligonucleotides of the present invention. The composition may be formulated with pharmaceutically acceptable additives as in the case with the composition of the present invention containing the oligomer of the present invention. A concentration of the expression vector contained in the composition may vary depending upon type of the career, etc., and is appropriately in a range of 0.1 nM to 100 μM, preferably in a range of 1 nM to 10 μM, and more preferably in a range of 10 nM to 1 μM. A weight ratio of the expression vector contained in the composition and the carrier (carrier/expression vector) may vary depending on property of the expression vector, type of the carrier, etc., and is appropriately in a range of 0.1 to 100, preferably in a range of 1 to 50, and more preferably in a range of 10 to 20. The content of the carrier contained in the composition is the same as in the case with the composition of the present invention containing the oligomer of the present invention, and a method for producing the same is also the same as in the case with the composition of the present invention.

Hereinafter, the present invention will be described in more detail with reference to EXAMPLES and TEST EXAMPLES below, but is not deemed to be limited thereto.

EXAMPLES

[Reference Example 1] 4-{[(2S,6R)-(4-amido-2-oxopyrimidin-1-yl)-4-tritylmorpholin-2-yl] methoxy}-4-oxobutanoic acid laded onto aminomethyl polystyrene resin

Step 1: Production of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl] meth oxy}-4-oxobutanoic acid

Under argon atmosphere, 22.0 g of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide and 7.04 g of 4-dimethylaminopyridine (4-DMAP) were suspended in 269 mL of dichloromethane, and 5.76 g of succinic anhydride was added to the suspension, followed by stirring at room temperature for 3 hours. To the reaction solution was added 40 mL of methanol, and the mixture was concentrated under reduced pressure. The residue was extracted using ethyl acetate and 0.5M aqueous potassium dihydrogenphosphate solution. The resulting organic layer was washed sequentially with 0.5M aqueous potassium dihydrogenphosphate solution, water and brine in the order mentioned. The resulting organic layer was dried over sodium sulfate and concentrated under reduced pressure to give 25.9 g of the product.

Step 2: Production of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1-yl)-4-tritylmorpholin-2-yl] methoxy}-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin

After 23.5 g of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid was dissolved in 336 mL of pyridine (dehydrated), 4.28 g of 4-DMAP and 40.3 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were added to the solution. Then, 25.0 g of Aminomethyl Polystyrene Resin cross-linked with 1% DVB (manufactured by Tokyo Chemical Industry Co., Ltd., A1543) and 24 mL of triethylamine were added to the mixture, followed by shaking at room temperature for 4 days. After completion of the reaction, the resin was taken out by filtration. The resulting resin was washed sequentially with pyridine, methanol and dichloromethane in the order mentioned, and dried under reduced pressure. To the resulting resin were added 150 mL of tetrahydrofuran (dehydrate), 15 mL of acetic anhydride and 15 mL of 2,6-lutidine, and the mixture was shaken at room temperature for 2 hours. The resin was taken out by filtration, washed sequentially with pyridine, methanol and dichloromethane in the order mentioned, and dried under reduced pressure to give 33.7 g of the product.

The loading amount of the product was determined by measuring UV absorbance at 409 nm of the molar amount of the trityl per g resin using a known method. The loading amount of the resin was 397.4 μmol/g.

Conditions of UV Measurement

Device: U-2910 (Hitachi, Ltd.)
Solvent: methanesulfonic acid
Wavelength: 265 nm
ε Value: 45000

[Reference Example 2] 4-Oxo-4-{[2S,6R)-6-(6-oxo-2-[2-phenoxyacetamido]-1H-purin-9-yl)-4-tritylmorpholin-2-yl]methoxy}butanoic acid loaded onto 2-aminomethylpolystyrene resin

Step 1: Production of N^2-(phenoxyacetyl)guanosine

Guanosine, 100 g, was dried at 80° C. under reduced pressure for 24 hours. After 500 mL of pyridine (anhydrous) and 500 mL of dichloromethane (anhydrous) were added thereto, 401 mL of chlorotrimethylsilane was dropwise added to the mixture under an argon atmosphere at 0° C., followed by stirring at room temperature for 3 hours. The mixture was again ice-cooled and 66.3 g of phenoxyacetyl chloride was dropwise added thereto. Under ice cooling, the mixture was stirred for further 3 hours. To the reaction solution was added 500 mL of methanol, and the mixture

was stirred at room temperature overnight. The solvent was then removed by distillation under reduced pressure. To the residue was added 500 mL of methanol, and concentration under reduced pressure was performed 3 times. To the residue was added 4 L of water, and the mixture was stirred for an hour under ice cooling. The precipitates formed were taken out by filtration, washed sequentially with water and cold methanol and then dried to give 150.2 g of the objective compound (yield: 102%) (cf.: Org. Lett. (2004), Vol. 6, No. 15, 2555-2557).

Step 2: N-{9-[(2R,6S)-6-(hydroxymethyl)-4-morpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide p-toluenesulfonate

In 480 mL of methanol was suspended 30 g of the compound obtained in Step 1, and 130 mL of 2N hydrochloric acid was added to the suspension under ice cooling. Subsequently, 56.8 g of ammonium tetraborate tetrahydrate and 16.2 g of sodium periodate were added to the mixture in the order mentioned and stirred at room temperature for 3 hours. The reaction solution was ice cooled and the insoluble matters were removed by filtration, followed by washing with 100 mL of methanol. The filtrate and washing liquid were combined and the mixture was ice cooled. To the mixture was added 11.52 g of 2-picoline borane. After stirring for 20 minutes, 54.6 g of p-toluenesulfonic acid monohydrate was slowly added to the mixture, followed by stirring at 4° C. overnight. The precipitates were taken out by filtration and washed with 500 mL of cold methanol and dried to give 17.7 g of the objective compound (yield: 43.3%).

^{1}H NMR (δ, DMSO-d6): 9.9-9.2 (2H, br), 8.35 (1H, s), 7.55 (2H, m), 7.35 (2H, m), 7.10 (2H, d, J=7.82 Hz), 7.00 (3H, m), 5.95 (1H, dd, J=10.64, 2.42 Hz), 4.85 (2H, s), 4.00 (1H, m), 3.90-3.60 (2H, m), 3.50-3.20 (5H, m), 2.90 (1H, m), 2.25 (3H, s)

Step 3: Production of N-{9-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide

In 30 mL of dichloromethane was suspended 2.0 g of the compound obtained in Step 2, and 13.9 g of triethylamine and 18.3 g of trityl chloride were added to the suspension under ice cooling. The mixture was stirred at room temperature for an hour. The reaction solution was washed with saturated sodium bicarbonate aqueous solution and then with water, and dried. The organic layer was concentrated under reduced pressure. To the residue was added 40 mL of 0.2M sodium citrate buffer (pH 3)/methanol (1:4 (v/v)), and the mixture was stirred. Subsequently, 40 mL of water was added and the mixture was stirred for an hour under ice cooling. The mixture was taken out by filtration, washed with cold methanol and dried to give 1.84 g of the objective compound (yield: 82.0%).

Step 4: Production of 4-oxo-4-{[(2S,6R)-6-(6-oxo-2-[2-phenoxyacetamide]-1H-purin-9-yl)-4-tritylmorpholin-2-yl]methoxy}butanoic acid loaded onto aminomethyl polystyrene resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that N-{9-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of REFERENCE EXAMPLE 1.

[Reference Example 3] 4-{[(2S,6R)-6-(5-Methyl-2,4-dioxo-3,4-dihydropyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that 1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-5-methylpyrimidine-2,4(1H,3H)-dione was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of REFERENCE EXAMPLE 1.

[Reference Example 4] 1,12-Dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that 2-[2-(2-hydroxyethoxyl)ethoxy]ethyl 4-tritylpiperazine-1-carboxylic acid (the compound described in WO 2009/064471) was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide.

According to the descriptions in EXAMPLES 1 to 12 and REFERENCE EXAMPLES 1 to 3 below, various types of PMO shown by PMO Nos. 1-11 and 13-16 in TABLE 2 were synthesized. The PMO synthesized was dissolved in injectable water (manufactured by Otsuka Pharmaceutical Factory, Inc.). PMO No. 12 was purchased from Gene Tools, LLC.

TABLE 2

PMO No.	Target sequence in exon 53	Note	SEQ ID NO:
1	31-55	5' end: group (3)	SEQ ID NO: 4
2	32-53	5' end: group (3)	SEQ ID NO: 8
3	32-56	5' end: group (3)	SEQ ID NO: 11
4	33-54	5' end: group (3)	SEQ ID NO: 15
5	34-58	5' end: group (3)	SEQ ID NO: 25
6	36-53	5' end: group (3)	SEQ ID NO: 32
7	36-55	5' end: group (3)	SEQ ID NO: 34
8	36-56	5' end: group (3)	SEQ ID NO: 35
9	36-57	5' end: group (3)	SEQ ID NO: 36

TABLE 2-continued

PMO No.	Target sequence in exon 53	Note	SEQ ID NO:
10	53-57	5' end: group (3)	SEQ ID NO: 18
11	39-69	Sequence corresponding to H53A(+39 − 69) (cf. Table 1) in Non-Patent Document 5, 5' end: group (3)	SEQ ID NO: 38
12	30-59	Sequence corresponding to h53A30/1 (cf. Table 1) in Non-Patent Document 5, 5' end: group (2)	SEQ ID NO: 39
13	32-56	5' end: group (1)	SEQ ID NO: 11
14	36-56	5' end: group (1)	SEQ ID NO: 35
15	30-56	Sequence corresponding to h53A30/1 cf. Table 1) in Non-Patent Document 5 5' end: group (3)	SEQ ID NO: 39
16	23-47	Sequence corresponding to SEQ ID NO: 429 described in Patent Document 4, 5' end: group (3)	SEQ ID NO: 47

Example 1

PMO No. 8

4-{[(2S,6R)-6-(4-Benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid, loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 1), 2 g (800 μmol) was transferred to a reaction vessel, and 30 mL of dichloromethane was added thereto. The mixture was allowed to stand for 30 minutes. After the mixture was further washed twice with 30 mL of dichloromethane, the following synthesis cycle was started. The desired morpholino monomer compound was added in each cycle to give the nucleotide sequence of the title compound.

TABLE 3

Step	Reagent	Volume (mL)	Time (min)
1	deblocking solution	30	2.0
2	deblocking solution	30	2.0
3	deblocking solution	30	2.0
4	deblocking solution	30	2.0
5	deblocking solution	30	2.0
6	deblocking solution	30	2.0
7	neutralizing solution	30	1.5
8	neutralizing solution	30	1.5
9	neutralizing solution	30	1.5
10	neutralizing solution	30	1.5
11	neutralizing solution	30	1.5
12	neutralizing solution	30	1.5
13	dichloromethane	30	0.5
14	dichloromethane	30	0.5
15	dichloromethane	30	0.5
16	coupling solution B	20	0.5
17	coupling solution A	6+11	90.0
18	dichloromethane	30	0.5
19	dichloromethane	30	0.5
20	dichloromethane	30	0.5
21	capping solution	30	3.0
22	capping solution	30	3.0
23	dichloromethane	30	0.5
24	dichloromethane	30	0.5
25	dichloromethane	30	0.5

The deblocking solution used was a solution obtained by dissolving a mixture of trifluoroacetic acid (2 equivalents) and triethylamine (1 equivalent) in a dichloromethane solution containing 1% (v/v) ethanol and 10% (v/v) 2,2,2-trifluoroethanol to be 3% (w/v). The neutralizing solution used was a solution obtained by dissolving N,N-diisopropylethylamine in a dichloromethane solution containing 25% (v/v) 2-propanol to be 5% (v/v). The coupling solution A used was a solution obtained by dissolving the morpholino monomer compound in 1,3-dimethyl-2-imidazolidinone containing 10% (v/v) N,N-diisopropylethylamine to be 0.15M. The coupling solution B used was a solution obtained by dissolving N,N-diisopropylethylamine in 1,3-dimethyl-2-imidazolidinone to be 10% (v/v). The capping solution used was a solution obtained by dissolving 20% (v/v) acetic anhydride and 30% (v/v) 2,6-lutidine in dichloromethane.

The aminomethyl polystyrene resin loaded with the PMO synthesized above was recovered from the reaction vessel and dried at room temperature for at least 2 hours under reduced pressure. The dried PMO loaded onto aminomethyl polystyrene resin was charged in a reaction vessel, and 200 mL of 28% ammonia water-ethanol (1/4) was added thereto. The mixture was stirred at 55° C. for 15 hours. The aminomethyl polystyrene resin was separated by filtration and washed with 50 mL of water-ethanol (1/4). The resulting filtrate was concentrated under reduced pressure. The resulting residue was dissolved in 100 mL of a solvent mixture of 20 mM acetic acid-triethylamine buffer (TEAA buffer) and acetonitrile (4/1) and filtered through a membrane filter. The filtrate obtained was purified by reversed phase HPLC. The conditions used are as follows.

TABLE 4

Column	XTera MS18 (Waters, φ30 × 100 mm, 1CV = 200 mL)
Flow rate	60 mL/min
Column temperature	room temperature
Solution A	20 mM TEAA buffer
Solution B	CH_3CN
Gradient	(B) conc. 20 → 50%/5CV

Each fraction was analyzed and the product was recovered in 100 mL of acetonitrile-water (1/1), to which 200 mL of ethanol was added. The mixture was concentrated under reduced pressure. Further drying under reduced pressure gave a white solid. To the resulting solid was added 300 mL of 10 mM phosphoric acid aqueous solution to suspend the solid. To the suspension was added 10 mL of 2M phosphoric acid aqueous solution, and the mixture was stirred for 15 minutes. Furthermore, 15 mL of 2M sodium hydrate aqueous solution was added for neutralization. Then, 15 mL of 2M sodium hydroxide aqueous solution was added to make the mixture alkaline, followed by filtration through a membrane filter (0.45 μm). The mixture was thoroughly washed

with 100 mL of 10 mM sodium hydroxide aqueous solution to give the product as an aqueous solution.

The resulting aqueous solution containing the product was purified by an anionic exchange resin column. The conditions used are as follows.

TABLE 5

Column	Source 30Q (GE Healthcare) φ 40 × 150 mm, 1CV = 200 mL)
Flow rate	80 mL/min
Column temp.	room temperature
Solution A	10 mM sodium hydroxide aqueous solution
Solution B	10 mM sodium hydroxide aqueous solution, 1M sodium chloride aqueous solution
Gradient	(B) conc. 5 → 35%/15CV

Each fraction was analyzed (on HPLC) and the product was obtained as an aqueous solution. To the resulting aqueous solution was added 225 mL of 0.1M phosphate buffer (pH 6.0) for neutralization. The mixture was filtered through a membrane filter (0.45 μm). Next, ultrafiltration was performed under the conditions

TABLE 6

Filter	PELLICON2 MINI FILTER PLBC 3K Regenerated Cellulose, Screen Type C
Size	0.1 m^2

The filtrate was concentrated to give approximately 250 mL of an aqueous solution. The resulting aqueous solution was filtered through a membrane filter (0.45 μm). The aqueous solution obtained was freeze-dried to give 1.5 g of the objective compound as a white cotton-like solid.

ESI-TOF-MS Calcd.: 6924.82.
Found: 6923.54.

Example 2

PMO. No. 1

The title compound was produced in accordance with the procedure of EXAMPLE 1.

MALDI-TOF-MS Calcd.: 8291.96.
Found: 8296.24.

Example 3

PMO. No. 2

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 7310.13.
Found: 7309.23.

Example 4

PMO. No. 3

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 8270.94.
Found: 8270.55.

Example 5

PMO. No. 4

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-(2H)-yl)-4-trityl-morpholin-2-yl)methoxy)-4-oxobutanoic acid (REFERENCE EXAMPLE 3) loaded onto aminomethyl polystyrene resin was used as the starting material.

ESI-TOF-MS Calcd.: 7310.13.
Found: 7310.17.

Example 6

PMO. No. 5

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-trityl-morpholin-2-yl)methoxy)-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 3) was used as the starting material.

ESI-TOF-MS Calcd.: 8270.94.
Found: 8270.20.

Example 7

PMO. No. 6

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 5964.01.
Found: 5963.68.

Example 8

PMO. No. 7

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 6609.55.
Found: 6608.85.

Example 9

PMO. No. 9

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-oxo-4-(((2S,6R)-6-(6-oxo-2-(2-phenoxyacetamido)-1H-purin-9(6H)-yl)-4-tritylmorpholin-2-yl)methoxy)butanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 2) was used as the starting material.

ESI-TOF-MS Calcd.: 7280.11.
Found: 7279.4.

Example 10

PMO. No. 10

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-oxo-4-(((2S,6R)-

6-(6-oxo-2-(2-phenoxyacetamido)-1H-purin-9(6H)-yl)-4-tritylmorpholin-2-yl)methoxy)butanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 2) was used as the starting material.

ESI-TOF-MS Calcd.: 8295.95.

Found: 8295.91.

Example 11

PMO. No. 13

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 1,12-dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 4) was used as the starting material.

ESI-TOF-MS Calcd.: 7276.15.

Found: 7276.69.

Example 12

PMO. No. 14

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 1,12-dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-5-pentadecanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 4) was used as the starting material.

ESI-TOF-MS Calcd.: 8622.27.

Found: 8622.29.

Comparative Example 1

PMO. No. 11

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 10274.63.

Found: 10273.71.

Comparative Example 2

PMO. No. 15

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 9941.33.

Found: 9940.77.

Comparative Example 3

PMO. No. 16

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 8238.94.

Found: 8238.69.

Test Example 1

In Vitro Assay

Using an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza), 10 uM of the oligomers PMO Nos. 1 to 8 of the present invention and the antisense oligomer PMO No. 11 were transfected with 4×10^5 of RD cells (human rhabdomyosarcoma cell line). The Program T-030 was used.

After transfection, the cells were cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen) under conditions of 37° C. and 5% CO_2. The cells were washed twice with PBS (manufactured by Nissui, hereinafter the same) and 500 μl of ISOGEN (manufactured by Nippon Gene) was added to the cells. After the cells were allowed to stand at room temperature for a few minutes to lyse the cells, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a Titan One Tube RT-PCR Kit (manufactured by Roche). A reaction solution was prepared in accordance with the protocol attached to the kit. A PTC-100 (manufactured by MJ Research) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

94° C., 2 mins: thermal denaturation

[94° C., 10 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

Forward primer:

(SEQ ID NO: 40)

5'-AGAATTTCGAACAGAGGCGTC-3'

Reverse primer:

(SEQ ID NO: 41)

5'-GTCTCCCACTGCCGAGGTC-3'

Next, a nested PCR was performed with the product amplified by RT-PCR above using a Taq DNA Polymerase (manufactured by Roche). The PCR program used is as follows.

94° C., 2 mins: thermal denaturation

[94° C., 15 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above are given below.

Forward primer:

(SEQ ID NO: 42)

5'-CATCAAGCAGAACGCAACAA-3'

Reverse primer:

(SEQ ID NO: 43)

5'-GAAGTTTCAGGGCCAAGTCA-3'

The reaction product, 1 μl, of the nested PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

Skipping efficiency (%)=$A/(A+B)\times100$

Experimental Results

The results are shown in FIG. 1. This experiment revealed that the oligomers PMO Nos. 1 to 8 of the present invention

caused exon 53 skipping with a markedly high efficiency as compared to the antisense oligomer PMO No. 11. In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than four times higher exon skipping efficiency than that of the antisense oligomer PMO No. 11.

Test Example 2

In Vitro Assay Using Human Fibroblasts

Human myoD gene (SEQ ID NO: 44) was introduced into TIG-119 cells (human normal tissue-derived fibroblasts, National Institute of Biomedical Innovation) or 5017 cells (human DMD patient-derived fibroblasts, Coriell Institute for Medical Research) using a ZsGreen1 coexpression retroviral vector.

After incubation for 4 to 5 days, ZsGreen-positive MyoD-transformed fibroblasts were collected by FACS and plated at 5×10^4/cm^2 into a 12-well plate. As a growth medium, there was used 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen Corp.) containing 10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

The medium was replaced 24 hours later by differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). The medium was exchanged every 2 to 3 days and incubation was continued for 12 to 14 days to differentiate into myotubes.

Subsequently, the differentiation medium was replaced by a differentiation medium containing 6 μM Endo-Porter (Gene Tools), and the morpholino oligomer was added thereto in a final concentration of 10 μM. After incubation for 48 hours, total RNA was extracted from the cells using a TRIzol (manufactured by Invitrogen Corp.). RT-PCR was performed with 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. A reaction solution was prepared in accordance with the protocol attached to the kit. An iCycler (manufactured by Bio-Rad) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 1 mins; 60° C., 1 mins; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 7 mins: final extension

The primers used were hEX51F and hEX55R.

hEX51F:
(SEQ ID NO: 45)
5'-CCGCCTTGCAACAGAACTTAC-3'

hEx55R:
(SEQ ID NO: 46)
5'-TCCTTACGGGTAGCATCCTG-3'

The reaction product of RT-PCR above was separated by 2% agarose gel electrophoresis and gel images were captured with a GeneFlash (Syngene). The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured using an Image J (manufactured by National Institutes of Health). Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation.

Skipping efficiency (%)=A/(A+B)×100

Experimental Results

The results are shown in FIGS. 2 and 3. This experiment revealed that in TIG-119 cells, the oligomers PMO Nos. 3, 8 and 9 of the present invention (FIG. 2) all caused exon 53 skipping with a higher efficiency than the antisense oligomer PMO No. 12 (FIG. 2). In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than twice higher exon skipping efficiency than that of the antisense oligomer PMO No. 12 (FIG. 2).

Furthermore, this experiment revealed that the oligomers PMO Nos. 3 and 8 to 10 of the present invention (FIG. 3) all caused exon 53 skipping with a higher efficiency than the antisense oligomer PMO No. 12 (FIG. 3). In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than seven times higher exon skipping efficiency than that of the antisense oligomer PMO No. 12 (FIG. 3).

Test Example 3

In Vitro Assay Using Human Fibroblasts

The skin fibroblast cell line (fibroblasts from human DMD patient (exons 45-52 or exons 48-52)) was established by biopsy from the medial left upper arm of DMD patient with deletion of exons 45-52 or DMD patient with deletion of exons 48-52. Human myoD gene (SEQ ID NO: 44) was introduced into the fibroblast cells using a ZsGreen1 coexpression retroviral vector.

After incubation for 4 to 5 days, ZsGreen-positive MyoD-transformed fibroblasts were collected by FACS and plated at 5×10^4/cm^2 into a 12-well plate. As a growth medium, there was used 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen Corp.) containing 10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

The medium was replaced 24 hours later by a differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). The medium was exchanged every 2 to 3 days and incubation was continued for 12, 14 or 20 days to differentiate into myotubes.

Subsequently, the differentiation medium was replaced by a differentiation medium containing 6 μM Endo-Porter (Gene Tools), and a morpholino oligomer was added thereto at a final concentration of 10 μM. After incubation for 48 hours, total RNA was extracted from the cells using a TRIzol (manufactured by Invitrogen Corp.). RT-PCR was performed with 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. A reaction solution was prepared in accordance with the protocol attached to the kit. An iCycler (manufactured by Bio-Rad) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 1 mins; 60° C., 1 mins; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 7 mins: final extension

The primers used were hEx44F and h55R.

hEx44F:
(SEQ ID NO: 48)
5'-TCTTGACAAATGGCGGCGT-3'

hEx55R:
(SEQ ID NO: 46)
5'-TCCTTACGGGTAGCATCCTG-3'

The reaction product of RT-PCR above was separated by 2% agarose gel electrophoresis and gel images were captured with a GeneFlash (Syngene). The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured using an Image J (manufactured by National Institutes of Health). Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation.

Skipping efficiency (%)=A/(A+B)×100

Experimental Results

The results are shown in FIGS. 4 and 5. This experiment revealed that the oligomers PMO Nos. 3 and 8 of the present invention caused exon 53 skipping with an efficiency as high as more than 80% in the cells from DMD patient with deletion of exons 45-52 (FIG. 4) or deletion of exons 48-52 (FIG. 5). Also, the oligomers PMO Nos. 3 and 8 of the present invention were found to cause exon 53 skipping with a higher efficiency than that of the antisense oligomer PMO No. 15 in the cells from DMD patient with deletion of exons 45-52 (FIG. 4).

Test Example 4

Western Blotting

The oligomer PMO No. 8 of the present invention was added to the cells at a concentration of 10 μM, and proteins were extracted from the cells after 72 hours using a RIPA buffer (manufactured by Thermo Fisher Scientific) containing Complete Mini (manufactured by Roche Applied Science) and quantified using a BCA protein assay kit (manufactured by Thermo Fisher Scientific). The proteins were electrophoresed in NuPAGE Novex Tris-Acetate Gel 3-8% (manufactured by Invitrogen) at 150V for 75 minutes and transferred onto a PVDF membrane (manufactured by Millipore) using a semi-dry blotter. The PVDF membrane was blocked with a 5% ECL Blocking agent (manufactured by GE Healthcare) and the membrane was then incubated in a solution of anti-dystrophin antibody (manufactured by NCL-Dys1, Novocastra). After further incubation in a solution of peroxidase-conjugated goat-antimouse IgG (Model No. 170-6516, Bio-Rad), the membrane was stained with ECL Plus Western blotting system (manufactured by GE Healthcare).

Immunostaining

The oligomer PMO No. 3 or 8 of the present invention was added to the cells. The cells after 72 hours were fixed in 3% paraformaldehyde for 10 minutes, followed by incubation in 10% Triton-X for 10 minutes. After blocking in 10% goat serum-containing PBS, the membrane was incubated in a solution of anti-dystrophin antibody (NCL-Dys1, Novocastra). The membrane was further incubated in a solution of anti-mouse IgG antibody (manufactured by Invitrogen). The membrane was mounted with Pro Long Gold Antifade reagent (manufactured by Invitrogen) and observed with a fluorescence microscope.

Experimental Results

The results are shown in FIGS. 6 and 7. In this experiment it was confirmed by western blotting (FIG. 6) and immunostaining (FIG. 7) that the oligomers PMO Nos. 3 and 8 of the present invention induced expression of the dystrophin protein.

Test Example 5

In Vitro Assay Using Human Fibroblasts

The experiment was performed as in TEST EXAMPLE 3.

Experimental Results

The results are shown in FIG. 8. This experiment revealed that in the cells from DMD patients with deletion of exons 45-52, the oligomers PMO Nos. 3 to 8 of the present invention caused exon 53 skipping with a higher efficiency than the oligomers PMO Nos. 13 and 14 of the present invention (FIG. 8).

Test Example 6

In Vitro Assay

Experiments were performed using the antisense oligomers of 2'-O-methoxy-phosphorothioates (2'-OMe-S-RNA) shown by SEQ ID NO: 49 to SEQ ID NO: 123. Various antisense oligomers used for the assay were purchased from Japan Bio Services. The sequences of various antisense oligomers are given below.

TABLE 7

Antisense oligomer	Nucleotide sequence	SEQ ID NO:
H53_39-69	CAUUCAACUGUUGCCUCCGGUUCUGAAGGUG	49
H53_1-25	UCCCACUGAUUCUGAAUUCUUUCAA	50
H53_6-30	CUUCAUCCCACUGAUUCUGAAUUCU	51
H53_11-35	UUGUACUUCAUCCCACUGAUUCUGA	52
H53_16-40	UGUUCUUGUACUUCAUCCCACUGAU	53
H53_21-45	GAAGCUGUCUCUUGUACUUCAUCCCA	54
H53_26-50	GUUCUGAAGCUGUCUCUUGUACUUCA	55
H53_31-55	CUCCGGUUCUGAAGCUGUCUCUUGUA	56
H53_36-60	GUUGCUUCCGGUUCUGAAGCUGUCU	57
H53_41-65	CAACUGUUGCCUCCGGUUCUGAAGU	58
H53_46-70	UCAUUCAACUGUUGCCUCCGGUUCU	59
H53_51-75	ACAUUUCAUUCAACUGUUGCCUCCG	60
H53_56-80	CUUUAACAUUUCAUUCAACUGUUGC	61
H53_61-85	GAAUCCUUUAACAUUUCAUUCAACU	62
H53_66-90	GUGUUGAAUCCUUUAACAUUUCAUU	63
H53_71-95	CCACUGUGUUCUAAUCCUUUAACAUU	64
H53_76-100	UCCAGCCAUUGUGUUGAAUCCUUUA	65
H53_81-105	UAGCUUCCAGCCAUUGUGUUGAAUC	66
H53_86-110	UUCCUUAGCUUCCAGCCAUUGUGUU	67
H53_91-115	CCUUCUUCCUUAGCUUCCAGCCAUU	68

TABLE 7-continued

Antisense oligomer	Nucleotide sequence	SEQ ID NO.
H53_96-120	GCUCAGCUUCUUCCUUACUUUCCAG	69
H53_101-125	GACCUGCUCAGCUCCUUCUUUAGCU	70
H53_106-130	CCUAAUACCUGCUCAGCUUCUUCCU	71
H53_111-135	CCUGUCCUAAGACCUGCUCAGCUUC	72
H53_116-140	UCUGGCCUGUCCUAAGACCUGCUCA	73
H53_121-145	UUGGCUCUGGCCUGUCCUAAUACCU	74
H53_126-150	CAAGCUUGGCUCUGGCCUGUCCUAA	75
H53_131-155	UGACUCAAGCUUGGCUCUGGCCUGU	76
H53_136-160	UUCCAUCAACUCAAGCUUGGCUCUG	77
H53_141-165	CCUCCUUCCAUGACUCAAGCUUGGC	78
H53_146-170	GGGACCCUCCUUCCAUGACUCAAGC	79
H53_151-175	GUAUAGGUACCCUCCUUCCAUGACU	80
H53_156-180	CUACUGUAUAUAGGUACCCUCCUUCCA	81
H53_161-185	UGCAUCUACUGUAUAGGUACCCUCC	82
H53_166-190	UGGAUUGCAUCUACUGUAUAGGUAC	83
H53_171-195	UCUUUUGGAUUGCAUCUACUGUAUA	84
H53_176-200	CACUUUCUUUUGGAUUGCAUCUACU	85
H53_181-205	UCUGUGAUUUUCUUUUGGAUUGCAU	86
H53_186-210	UGGUUUCUGUGAUUUUCUUUUGGAU	87
H53_84-108	CCUUAGCUUCCAGCCAUUGUGUGA	88
H53_88-112	UCUUCCUUACUUCCAGCCAUUGUG	89
H53_119-143	GGCUCUGGCCUGUCCUAAGACCUGC	90
H53_124-148	AGCUUGGCUCUGGCCUGUCCUAAGA	91
H53_128-152	CUCAAGCUUGGCUCUGGCCUGUCCU	92
H53_144-168	CACCCUCCUUCCAUGACUCAAGCUU	93
H53_149-173	AUACGGACCCUCCUUCCAUGACUCA	94
H53_153-177	CUGUAUAGGUACCCUCCUUCCAUGCA	95
H53_179-203	UGUGAUUUUCUUUUGGAUUGCAUCU	96
H53_184-208	GUUUCUGUGAUUUUCUUUUGGAUUG	97
H53_188-212	CUUGGUUUCUGUGAUUUUCUUUUGG	98
H53_29-53	CCGGUUCUGAAGGUGUUCUUGUACU	99
H53_30-54	UCCGGUUCUGAAGGUGUUCUUGUAC	100
H53_32-56	CCUCCGGUUCUGAAGGUGUUCUUGU	101
H53_33-57	CCUCCGGUUCUGAAGGUGUUCUUGU	102
H53_34-58	UCCUCCGGUUCUGAAGGUGUUCUU	103
H53_35-59	UUCCUCCGGUUCUGAAGGUGUUCU	104
H53_37-61	UGUUCCUCCGGUUCUGAAGGUGUU	105
H53_38-62	CUCUUCCUCCGGUUCUGAAGGUGU	106
H53_39-63	ACUGUUCCUCCGGUUCUGAAGGUG	107

TABLE 7-continued

Antisense oligomer	Nucleotide sequence	SEQ ID NO.
H53_30-54	AACUGUUGCCUCCGGUUCUGAAGGU	108
H53_32-61	UGUUGCCUCCGGUUCUGAAGGUGUUCUUGU	109
H53_32-51	GGUUCUGAAGGUGUUCUUGU	110
H53_35-54	UCCGGUUCUGAAGGUGUUCU	111
H53_37-56	CCUCCGGUUCUGAAGGUGUU	112
H53_30-59	UUGCCUCCGGUUCUGAAGGU	113
H53_32-61	UGUUGCCUCCGGUUCUGAAG	114
H53_32-49	UUCUGAAGGUGUUCUUGU	115
H53_35-52	CGGUUCUGAAGGUGUUCU	116
H53_38-55	CUCCGGUUCUGAAGGUGU	117
H53_41-58	UGCCUCCGGUUCUGAAGG	118
H53_44-61	UGUUGCCUCCGGUUCUCA	119
H53_35-49	UUCUGAAGGUGUUCU	120
H53_40-54	UCCGGUUCUGAAGGU	121
H53_45-59	UUCCUCCGGUUCUG	122
H53_45-62	CUGUUGCCUCCGGUUCUG	123

RD cells (human rhabdomyosarcoma cell line) were plated at 3×10^5 in a 6-well plate and cultured in 2 ml, of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, Inc., hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen Corp.) under conditions of 37° C. and 5% CO_2 overnight. Complexes of various antisense oligomers (Japan Bio Services) (1 μM) for exon 53 skipping and Lipofectamine 2000 (manufactured by Invitrogen Corp.) were prepared and 200 μl was added to RD cells where 1.8 mL of the medium was exchanged, to reach the final concentration of 100 nM.

After completion of the addition, the cells were cultured overnight. The cells were washed twice with PBS (manufactured by Nissui, hereafter the same) and then 500 μl of ISOGEN (manufactured by Nippon Gene) were added to the cells. After the cells were allowed to stand at room temperature for a few minutes for cell lysis, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a Titan One Tube RT-PCR Kit (manufactured by Roche). A reaction solution was prepared in accordance with the protocol attached to the kit. A PTC-100 (manufactured by MJ Research) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

94° C., 2 mins: thermal denaturation

[94° C., 10 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

Forward primer:
(SEQ ID NO: 42)
5'-CATCAAGCAGAAGGCAACAA-3'

Reverse primer:
(SEQ ID NO: 43)
5'-GAAGTTTCAGGCCAAGTCA-3'

Subsequently, a nested PCR was performed with the amplified product of RT-PCR above using a Taq DNA Polymerase (manufactured by Roche). The PCR program used is as follows.

94° C., 2 mins: thermal denaturation

[94° C., 15 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above are given below.

Forward primer:
(SEQ. ID NO: 40)
5'-AGGATTTGGAACAGAGGCGTC-3'

Reverse primer:
(SEQ ID NO: 41)
5'-GTCTGCCACTGGCGGAGGTC-3'

The reaction product, 1 μl, of the nested PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

Skipping efficiency (%)=$A/(A+B)\times 100$

Experimental Results

The results are shown in FIGS. 9 to 17. These experiments revealed that, when the antisense oligomers were designed at exons 31-61 from the 5' end of exon 53 in the human dystrophin gene, exon 53 skipping could be caused with a high efficiency.

Test Example 7

Using an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza), 0.3 to 30 μM of the antisense oligomers were transfected with 3.5×10^5 of RD cells (human rhabdomyosarcoma cell line). The Program T-030 was used.

After the transfection, the cells were cultured overnight in 2 ml of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, Inc., hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen Corp.) under conditions of 37° C. and 5% CO_2. The cells were washed twice with PBS (manufactured by Nissui, hereinafter the same) and 500 μl of ISOGEN (manufactured by Nippon Gene) was then added to the cells. After the cells were allowed to stand at room temperature for a few minutes to lyse the cells, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit (manufactured by Qiagen, Inc.). A reaction solution was prepared in accordance with the protocol attached to the kit. The thermal cycler used was a PTC-100 (manufactured by MJ Research). The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 30 seconds; 60° C., 30 seconds; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 10 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

Forward primer:
(SEQ. ID NO: 42)
5'-CATCAAGCAGAAGGCAACAA-3'

Reverse primer:
(SEQ. ID NO: 43)
5'-GAAGTTTCAGGCCAAGTCA-3'

The reaction product, 1 μl, of the PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

Skipping efficiency (%)=$A/(A+B)\times 100$

Experimental Results

The results are shown in FIGS. 18 and 19. These experiments revealed that the oligomer PMO No. 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the antisense oligomers PMO Nos. 15 and 16 (FIG. 18). It was also revealed that the oligomers PMO Nos. 3 and 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the oligomers PMO Nos. 13 and 14 of the present invention (FIG. 19). These results showed that the sequences with —OH group at the 5' end provide a higher skipping efficiency even in the same sequences.

INDUSTRIAL APPLICABILITY

Experimental results in TEST EXAMPLES demonstrate that the oligomers of the present invention (PMO Nos. 1 to 10) all caused exon 53 skipping with a markedly high efficiency under all cell environments, as compared to the oligomers (PMO Nos. 11, 12, 15 and 16) in accordance with the prior art. The 5017 cells used in TEST EXAMPLE 2 are the cells isolated from DMD patients, and the fibroblasts used in TEST EXAMPLES 3 and 5 are exon 53 skipping target cells from DMD patients. Particularly in TEST EXAMPLES 3 and 5, the oligomers of the present invention show the exon 53 skipping efficiency of 80% or higher in the cells from DMD patients that are the target for exon 53 skipping. Consequently, the oligomers of the present invention can induce exon 53 skipping with a high efficiency, when DMD patients are administered.

Therefore, the oligomers of the present invention are extremely useful for the treatment of DMD.

Sequence Listing Free Text

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SEQ ID NO: 5: synthetic nucleic acid
SEQ ID NO: 6: synthetic nucleic acid
SEQ ID NO: 7: synthetic nucleic acid
SEQ ID NO: 8: synthetic nucleic acid

SEQ ID NO: 9: synthetic nucleic acid
SEQ ID NO: 10: synthetic nucleic acid
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Sequence Listing

SEQUENCE LISTING

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cctccggttc tgaaggtgtt cttgta 26

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ccggttctga aggtgttcct gt 22

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tccggttctg aaggtgttcc tgt 23

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ctccggttct gaaggtgttc ctgt 24

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tgcctcccgt tgtgaaggtg ttcttgt 27

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ccggttctga aggtgttctt g 21

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tccggttctg aaggtgttct tg 22

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ctccggttct gaaggtgttc ttg 23

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cctccggttc tgaaggtgtt cttg 24

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gcctccggtt ctgaaggtgt tcttg 25

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cctccggttc tgaaggtgtt ctt 23

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gcctccggtt ctgaaggtgt tctt 24

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ccggttctga aggtgttct 19

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tccggttctg aaggtgttct 20

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ctccggttct gaaggtgttc t 21

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<400> SEQUENCE: 29

cctccggttc tgaaggtgtt ct 22

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gcctccggtt ctgaaggtgt tct 23

<210> SEQ ID NO 31

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<211> LENGTH: 24
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<400> SEQUENCE: 31

tgcctccggt tctgaaggtg ttct 24

<210> SEQ ID NO 32
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<400> SEQUENCE: 32

cggttctga aggtgttc 16

<210> SEQ ID NO 33
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial
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<400> SEQUENCE: 33

tcggttctg aaggtgttc 19

<210> SEQ ID NO 34
<211> LENGTH: 20
<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 34

ctcggttct gaaggtgttc 20

<210> SEQ ID NO 35
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 35

cctccggttc tgaaggtgtt c 21

<210> SEQ ID NO 36
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 36

gcctccggtt ctgaaggtgt tc 22

<210> SEQ ID NO 37
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<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 37

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tgcctcggt tctgtaggtg ttc 23

<210> SEQ ID NO 38
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<213> ORGANISM: Artificial
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<400> SEQUENCE: 38

catcaaacag ttgccctcgg ttctgtaggt g 31

<210> SEQ ID NO 39
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<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 39

ttgcctcggg ttctgtaggt gttctgccac 30

<210> SEQ ID NO 40
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<212> TYPE: DNA
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<400> SEQUENCE: 40

aggattggga acagaggcgt c 21

<210> SEQ ID NO 41
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 41

gtctgccact ggcggaaggc 20

<210> SEQ ID NO 42
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 42

catcaagcag aaggcatcaa 20

<210> SEQ ID NO 43
<211> LENGTH: 20
<212> TYPE: DNA
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<400> SEQUENCE: 43

gaaagtttcag ggccaagtca 20

<210> SEQ ID NO 44
<211> LENGTH: 863
<212> TYPE: DNA

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<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 44

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cgcttcttcg atgacctgga ccgcgccctg atgcacgtgg gcgccgtcct gaaaccccgc 180

gagcactcgc actccccgc ggcggtgcac ccggccccgg gcgccacgtga gtacgacgcat 240

gtgcgcgcgc ccagcgggca cgaccaggcg ggcacgtgcc tactgtgggc ctgcaaggcg 300

tgcaagcgca agaccaccaa cgccgtcgtc cgcaaggccg ccaccatgcg cgagcggcgc 360

cgcctgagca aagtaaatga ggctttgaaj acaatcaagc gctgcacgtc gagcaatccc 420

aaccagcggt tgtccaaggt ggagatcctg cgcaacgccc tccgctatat cgagggcctg 480

caggatctgc tgcgcgacca ggacgcgcg cccctggcg ccgcagccgc cttctatgcg 540

ccgggccgcc tgcccccggg cgcgcggcgc gagcactaca gcggcgactc cgacgcgtcc 600

agcccgcgct ccaactgctc cgacggcatg atggactacg gcggcccccc gagcggcgcc 660

cggcggcgga actgctacga aggcgcctac tacaacgagg cgcccagcga acccgggccc 720

gggaagagtg cggcggtgtc gagcctagac tgcctgtcca gcatcgtgga gcgcatctcc 780

accgagaagc ctgcggcgcc cgccctcctg ctggcgacg tgcctttcga gtagcctccg 840

cgcaggcaag acgctgccgc cccagcgag ggagagagca gcggcgaccc caccagtca 900

ccggacgccg cccgcagtg cctgcgggt gcgaaccccA accgatata ccaggtgctc 960

tga 963

<210> SEQ ID NO 45
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<212> TYPE: DNA
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<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 45

cgcgcttgga cagaacttac 20

<210> SEQ ID NO 46
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
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<400> SEQUENCE: 46

tccttacggg tagcatcctg 20

<210> SEQ ID NO 47
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 47

ctgaagtgtt tcttgtactt catcc 25

<210> SEQ ID NO 48
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial

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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 48

tgttgagaaa tggcggcgt 19

<210> SEQ ID NO 49
<211> LENGTH: 31
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 49

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<210> SEQ ID NO 50
<211> LENGTH: 25
<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 50

uccacugau ucugaauucu uucaa 25

<210> SEQ ID NO 51
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 51

cuucaaccca cugaauucug agucu 25

<210> SEQ ID NO 52
<211> LENGTH: 25
<212> TYPE: RNA
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<220> FEATURE:
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<400> SEQUENCE: 52

uagacuuca uccacugau ucuga 25

<210> SEQ ID NO 53
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 53

ugaucugaa cuucaaccca cugau 25

<210> SEQ ID NO 54
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 54

gaaggugaac uagacuuca uccca 25

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<210> SEQ ID NO 55
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 55

guucugaagg uguucuugua cuucu 25

<210> SEQ ID NO 56
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 56

cuccgguucu gaagguguuc uugua 25

<210> SEQ ID NO 57
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 57

guugcacucg guucugaagg uguuc 25

<210> SEQ ID NO 58
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 58

caacuguugc cuccgguucu gaagg 25

<210> SEQ ID NO 59
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<212> TYPE: RNA
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<400> SEQUENCE: 59

ucauucaaca guugcacucg guucu 25

<210> SEQ ID NO 60
<211> LENGTH: 25
<212> TYPE: RNA
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<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 60

acauuucaau caacuguugc cuccg 25

<210> SEQ ID NO 61
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<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

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<400> SEQUENCE: 61

ccuuacauu ucauucaaca guugc 25

<210> SEQ ID NO 62
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 62

gauuccuuua acauuucauu caacu 25

<210> SEQ ID NO 63
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 63

guguugaauc cuuuaacauu ucauu 25

<210> SEQ ID NO 64
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 64

ccaauguguu gauuccuuua acauu 25

<210> SEQ ID NO 65
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 65

uccaggcaua guguugaauc cuuua 25

<210> SEQ ID NO 66
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 66

uagcuuccag ccaauguguu gauuc 25

<210> SEQ ID NO 67
<211> LENGTH: 25
<212> TYPE: RNA
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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 67

uuccuuagcu uccaggcaua guguu 25

<210> SEQ ID NO 68

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<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 68

gcuucuuccu usgcuucag ccauu 25

<210> SEQ ID NO 69
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<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 69

gcucagcuuc uuccuiagcu uccag 25

<210> SEQ ID NO 70
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 70

gaccugcuca gcuucuuccu uagcu 25

<210> SEQ ID NO 71
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 71

ccuaagaccu gcucagcuuc uuccu 25

<210> SEQ ID NO 72
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 72

ccuguccuaa gaccugcuca gcuuc 25

<210> SEQ ID NO 73
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 73

ucuggccugu ccuaagaccu gcuca 25

<210> SEQ ID NO 74
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 74

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uuggcucugg ccuguccuaa gacca 25

<210> SEQ ID NO 75
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 75

caagcuugga ucuggccugu ccuaa 25

<210> SEQ ID NO 76
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 76

ugacucaagc uuggcucugg ccugu 25

<210> SEQ ID NO 77
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 77

uuccaugacu caagcuugga ucugg 25

<210> SEQ ID NO 78
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 78

ccucccucca ugacucaagc uugga 25

<210> SEQ ID NO 79
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 79

ggaccccucc uuccaugacu caagc 25

<210> SEQ ID NO 80
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

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<400> SEQUENCE: 80

guauaggguc cuuccuucca ugacu 35

<210> SEQ ID NO 81
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 81

cuacuguaua gggaccuucc uucca 25

<210> SEQ ID NO 82
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 82

ugcaucuacu guauagggac ccucc 25

<210> SEQ ID NO 83
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 83

uggauugcau cuacuguaua gggac 25

<210> SEQ ID NO 84
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 84

ucuauuggau ugcaucuacu guaua 25

<210> SEQ ID NO 85
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 85

gauauucuuu uggauugcau cuacu 25

<210> SEQ ID NO 86
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 86

ucugugauuu ucuauuggau ugcau 25

<210> SEQ ID NO 87

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<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 87

ugggaucugu gauuauuuuu uggau 25

<210> SEQ ID NO 88
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 88

ccuuagcuuc cagccauugu guuga 25

<210> SEQ ID NO 89
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 89

ucuuccuuag cuuccagcca uugug 25

<210> SEQ ID NO 90
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 90

ggcucuggac uguccuauga caugc 25

<210> SEQ ID NO 91
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 91

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uucugaaggu guucu 15

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ugccuccagg uucug 15

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cuguugccuc aggucucug 18

The invention claimed is:

1. An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of the nucleotide sequence of SEQ ID NO: 57, wherein the antisense oligomer is an oligonucleotide in which the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified, or a morpholino oligomer.

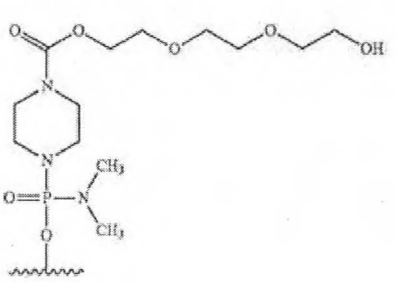
2. The antisense oligomer according to claim 1, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of: OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br, and I, wherein R is an alkyl or an aryl and R' is an alkylene.

3. The antisense oligomer according to claim 1, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of: a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond, and a boranophosphate bond.

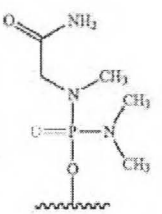
4. The antisense oligomer according to claim 1, which is a morpholino oligomer.

5. The antisense oligomer according to claim 4, which is a phosphorodiamidate morpholino oligomer.

6. The antisense oligomer according to claim 4, wherein the 5' end is any one of the groups of chemical formulae (1) to (3) below:



(1)



(2)

-continued

(3)

7. A pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the antisense oligomer according to claim 1, or a pharmaceutically acceptable salt or hydrate thereof.

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